CHARACTERISATION OF A NOVEL FORM OF PROTEIN KINASE C FROM ANTERIOR PITUITARY TISSUE

by

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I declare that this thesis has been composed by me and that the studies presented are the result of my own independent investigation, with the exception of the immunoblots, which were carried out with the assistance of Jim Simpson, the HAP fractionation and autophosphorylation, which were carried out with the assistance of Roger Clegg and some of the PKC activity assays and PCR amplification, which were carried out with the assistance of Melanie Johnson and Eve Lutz respectively.

This work has not been and is not currently being submitted for candidature in any other degree or professional qualification.

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Thomson, F.J., Johnson, M.S., Mitchell, R., Wolbers, W.B., Ison, A.J. and MacEwan, D.J. (1993) The differential effects of PKC activators and inhibitors on rat anterior pituitary hormone release. *Mol. Cell. Endocrinol.* **94**: 223-234.

ASTRACT

A number of cellular responses mediated by protein kinase C (PKC) in the anterior pituitary gland involve a form of PKC that is unusually resistant to the PKC inhibitor H7. In this study, inhibition by staurosporine, H7 and Ro 31-8220 of phorbol 12,13-dibutyrate-induced PKC activity in cytosol partially-purified from rat midbrain, anterior pituitary and a number of other tissues, as well as COS 7 cells, was studied. An *in vitro* mixed micelle histone IIIS phosphorylation assay was used to allow comparison of Ca^{2+}-dependent and Ca^{2+}-independent PKC activity. In anterior pituitary but not midbrain or COS 7 cells, Ca^{2+}-independent activity was notably resistant to H7 but sensitive to staurosporine and Ro 31-8220. All Ca^{2+}-dependent activity was sensitive to these three inhibitors. Comparison of PKC activity from a variety of different tissue sources showed H7-resistant, Ca^{2+}-independent activity also occurred in lung but not in spleen, thalamus or cerebellum. Mezerein and 1,2-dioctanoyl-*sn* -glycerol also activated this H7-insensitive PKC from anterior pituitary. Phosphorylation of a number of substrates by anterior pituitary PKCs were also compared. When [$Ala^{9,10}$, $Lys^{11,12}$] glycogen $synthase_{1-12}$ (GS peptide) but not $[Ser]^{25}$ PKC α_{19-31} was the phosphate acceptor, a clear component of the activity, which was Ca^{2+}-independent, was resistant to H7. The tissue distribution of this activity and its characteristic resistance to H7 but not other inhibitors, does not obviously correlate with that of any of the well-characterised PKCs, and may reflect either a novel or a modified isoform.

To further characterise this H7-resistant PKC, it was necessary to separate it from other pituitary PKCs. Initial fractionation studies showed that DEAE-cellulose chromatography can separate Ca^{2+}-independent PKC activity from anterior pituitary tissue into fractions with different sensitivities to

H7. Furthermore fractionation by hydroxyapatite (HAP) chromatography of both anterior pituitary and midbrain extracts resulted in three main peaks of PKC activity but only anterior pituitary contained significant PDBu-induced PKC activity which eluted after the third peak. This activity was not seen in other tissues such as spleen and COS 7 cells but was present in lung and αT3-1 cell extracts. Ca^{2+}-independent activity in this fraction from pituitary was sensitive to the PKC inhibitor Ro 31-8220 but relatively resistant to H7. H7-resistant activity was also detected in fractions of HAP eluate from lung and αT3-1 cells. Immunoblotting with antisera specific for PKC α, β_1, δ, ε, ζ and θ, isoforms present in pituitary, showed that this fraction did not contain the major immunoreactivity for any of these PKCs. Phosphatidylserine-dependent autophosphorylation of HAP fractions from anterior pituitary revealed the presence of a 140 kDa protein which was not seen in midbrain and whose distribution between the fractions correlated with the H7-resistant PKC activity. Similarly, immunoblotting with an antiserum raised to a PKC consensus sequence (in the catalytic domain) showed a prominent immunoreactive species at approximately 130 kDa which was present selectively in those HAP fractions from anterior pituitary and lung that displayed H7-resistant activity. Thus the 130 kDa protein may well represent the H7-resistant kinase, and may be related to one of the known PKCs or a novel isoform of PKC.

The possibility that a novel PKC isoform may be expressed in anterior pituitary was examined by polymerase chain reaction (PCR) amplification of reverse transcribed RNA from anterior pituitary and DNA from a rat pituitary cDNA library. This employed degenerate oligonucleotide primers corresponding to peptide sequences conserved between PKC isoforms but not other protein kinases to amplify partial core sequences of putative PKC genes. Analysis of clones using selected restriction endonucleases and

subsequent DNA sequencing analysis identified sequences encoding PKC α, ε and ζ but no novel PKC-related sequences. The possible reasons for this as well as alternative strategies yet to be employed are discussed in the light of PKC sequences published since the design of this study.

ABBREVIATIONS

A	alanine (peptide sequence)
A	adenine (nucleotide sequence)
Ac	acetate
aPKC	atypical protein kinase C
APS	ammonium persulphate
ATP	adenosine-5'-triphosphate
ATPase	adenosine-5'-triphosphatase
bp	base pair(s)
BSA	bovine serum albumin
C	cytosine
$C0_2$	carbon dioxide
Ca^{2+}	calcium ion
cAMP	adenosine 3', 5'-cyclic monophosphate
cDNA	complementary DNA
cGMP	cyclic guanosine monophosphate
Ci	curies
cm	centimetre
CNS	central nervous system
cPKC	classical protein kinase C
Cys	cysteine
Da	daltons
DAG	diacylglycerol
dATP	2'-deoxyadenosine
dCTP	2'-deoxycytosine
ddNTP	2', 3'-dideoxyribonucleoside 5'-triphosphate
DEAE	diethylaminoethyl
dGTP	2'-deoxyguanosine-5'-triphosphate
dH_2O	distilled water
DMEM	Dulbecco's modified Eagle's medium
DMF	dimethylformamide
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
dNTP	2'-deoxyribonucleoside 5'-triphosphate
dpm	disintegrations per minute
DRG	dorsal root ganglion (ganglia)
DTT	dithiothreitiol

dTTP	2'-deoxythymidine-5'-triphosphate
E	glutamic acid
E. coli	*Escherichia coli*
EDTA	sodium ethylenediamine tetra-acetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(b-aminoether)N,N,N',N'-tetraacetic acid
F	phenylalanine
G	glycine (peptide sequence)
G	guanine (nucleotide sequence)
g	gram(s)
G-protein	guanine nucleotide-binding protein
GH	growth hormone
Gly	glycine residue
GS	glycogen synthase
H3PO4	phosphoric acid
H7	1-(5-isoquinoline sulphonyl)-2-methyl-piperazine hydrochloride
HAP	hydroxyapatite
HCl	hydrochloric acid
I	isoleucine
IP_3	inositol 1,3,4 triphosphate
IPTG	isopropylthio-β-D-galactoside
K	lysine
kDa	kilo daltons
l	litre(s)
L	leucine
LH	luteinising hormone
LHRH	luteinising hormone-releasing hormone
LTP	long term potentiation
M	methionine (amino acid sequence)
M	molar
mA	milliamp(s)
MAP kinase	mitogen activated protein kinase
MARCKS	myristolated alanine-rich C kinase substrate
MBP	myelin basic protein
MEK	MAP kinase kinase
Mg^{2+}	magnesium ion
min	minute(s)

ml	milliletres
mM	millimolar
mMol	millimolar
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
N	asparagine
Na^+	sodium ion
ng	nanogram
nPKC	novel protein kinase C
OD	optical density
P	proline
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PCR	polymerase chain reaction
PDBu	phorbol 12, 13-dibutyrate
PI	phosphatidylinositol
PIP_2	phosphatidylinositol 4,5-biphosphate
PIP_3	phosphatidylinositol 3,4,5-triphosphate
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylemethylsulfonylflouide
PS	phosphatidylserine
Q	glutamine
R	arginine
RACK	receptor for activated C kinase
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RT	reverse transcriptase
S	serine
SDS	sodium dodecyl sulphate
sec	second
T	threonine (amino acid sequence)
T	thymidine (nucleotide sequence)

TBE	tris-borate/EDTA
TCA	trichloracetic acid
TEMED	N,N,N',N',-teramethylethylene diamine
T_m	melting temparature
TPA	tetradecanoylphorbolacetate
Tris	tris (hydroxymethyl) aminoethane
UV	ultraviolet
V	valine (amino acid sequence)
V	volt
v/v	volume/volume
W	tryptophan
w/v	weight/volume
X-gal	5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside
Y	tyrosine
^{32}P	phosphorous 32 radioisotope
^{35}S	sulphur 35 radioisotope

TABLE OF CONTENTS

CHAPTER 1

INTRODUCTION

Protein phosphorylation by protein kinases is a regulatory event essential to many cell signalling pathways. Protein kinase C (PKC) is a serine/threonine kinase that is involved in a wide variety of cellular responses, including neurotransmitter release, hormone secretion, cell growth and differentiation, and regulation of gene expression (Nishizuka, 1992). It was initially identified as a proteolytically-activated protein kinase (Inoue et al., 1977; Takai et al., 1977) that was later discovered to be Ca^{2+}-activated and dependent on phospholipids for its activity (Takai et al., 1979b). It was soon linked to signal transduction pathways following inositol phospholipid hydrolysis, a process that results in the production of diacylglycerol (DAG), which was found to activate PKC (Takai et al., 1979a). It is now known that DAG generated by other reactions is also able to activate PKC (Exton, 1990; Pelech and Vance, 1989), and this enzyme is the major cellular receptor for the tumour-promoting phorbol esters (Nishizuka, 1988). These compounds activate PKC in a manner similar to DAG (Castagna et al., 1982) and are widely used to mimic agonist-induced responses that are thought to involve PKC. Protein kinase C is now known to represent a family of related kinases which are structurally similar but vary in their Ca^{2+}-dependence and other biochemical properties (Nishizuka, 1988), and this may contribute to enabling PKC to play a role in such a wide variety of cellular responses (Hug and Sarre, 1993).

1.1 PKC ISOFORMS

Initially three different Ca^{2+}-dependent isoforms of PKC, PKC I, II and III, were identified by hydroxyapatite (HAP) purification of rat brain tissue (Huang et al., 1986b), although more isoforms were soon identified by molecular cloning studies (Ono et al., 1988). Eleven different isoforms have

been cloned to date (Johannes et al., 1994; Nishizuka, 1992) although it is possible that further PKCs may exist. These isoforms may be divided into three groups on the basis of their activation characteristics (Nishizuka, 1992). The classical PKCs (cPKCs), the first group to be identified, consist of PKC α, βI, βII and γ and are all dependent upon Ca^{2+} for activation. The novel or nPKCs (δ, ε, η and θ) are active in the absence of Ca^{2+} although these isoforms, like the cPKCs, require phorbol esters or DAG for activation. The third group, the atypical or aPKCs, currently has three members (PKC ζ, ι (λ) and μ) and these isoforms are not thought to be activated by either phorbol esters or Ca^{2+} (Johannes et al., 1994; McGlynn et al., 1992; Nakanishi and Exton, 1992; Selbie et al., 1993). These eleven isoforms are the product of ten different genes and are all structurally related, although the most recently identified isoform, PKC μ , is more distantly related to the other isoforms (Johannes et al., 1994).

1.2 PKC STRUCTURE

The PKC molecule has two domains, an N-terminal regulatory domain and a C-terminal catalytic domain, as is common to most single subunit protein kinases (Hanks et al., 1988), the two domains being linked by a flexible hinge region (Pears and Parker, 1991). From the sequence of the cDNAs it is clear that the cPKCs have four conserved domains (C1-4) and five variable regions (V1-5) (Coussens et al., 1986) as shown in Figure 1.1. The nPKCs and aPKCs lack the C2 domain. This domain is thought to be responsible for Ca^{2+} dependency of the cPKCs, although there is no evidence in this domain of a typical EF hand Ca^{2+} binding sequence as seen in other Ca^{2+} binding proteins (Huang, 1989). Deletion of this region in PKC γ results in a kinase that is capable of being activated by phorbol esters but is independent of Ca^{2+} (Ono et al., 1989a). There is now

evidence, from deletion mutants of PKC βI, to suggest that Ca^{2+} is bound mainly to the C1 region but the C2 domain confers specificity for Ca^{2+} binding in the cPKCs (Luo and Weinstein, 1993). Other divalent cations, eg Mg^{2+} which is present in cells at millimolar concentrations, are proposed to substitute for Ca^{2+} in activation of the nPKCs (Luo and Weinstein, 1993).

The C1 domain contains an autoinhibitory domain, as is seen in a number of other kinases (Soderling, 1990), and cysteine-rich regions similar to those seen in DNA binding proteins (Parker et al., 1989). The autoinhibitory or pseudosubstrate domain is fairly well conserved between the cPKCs but different in the nPKCs and cPKCs (Parker et al., 1989). All the PKC isoforms have a conserved distance of 15 amino acids between the end of the pseudosubstrate domain and the beginning of the first cysteine-rich region but the significance of this is unclear (Hug and Sarre, 1993). The cPKCs and nPKCs contain two cysteine rich regions while there is only one in the aPKCs ζ and ι (Ono et al., 1989b, Selbie et al., 1993). Protein kinase C μ also contains two cysteine rich regions although, unlike in the other PKC isoforms, they are separated by a long intervening sequence (Johannes et al., 1994). These cysteine rich regions are thought to be involved in phorbol ester binding (Burns and Bell, 1991) and the aPKCs ζ and ι contain only one such sequence and these isoforms are unable to binding phorbol esters (Liyanage et al., 1992; Selbie et al., 1993). However it has been shown that one of the cysteine regions alone is capable of binding these activators (Burns and Bell, 1991) and site-directed mutagenesis experiments have been conducted to determine the minimum requirements for phorbol binding (Quest et al., 1994). These regions are known to bind two zinc molecules and it has been suggested that this is essential for stabilising the structure involved in lipid interactions and

phorbol ester binding (Quest et al., 1992), although the precise relationship between zinc and phorbol ester binding at present remains unclear.

The C3 domain contains the consensus amino acid sequence G X G X X G, (X representing any amino acid), which is conserved in many protein kinases (Hanks et al., 1988) as well as nucleotide binding proteins (Wierenga and Hol, 1983) and from molecular modelling studies, this has been suggested to represent the ATP binding site (Sternberg and Taylor, 1984). This consensus sequence is altered in PKC ζ and ι, where the third glycine residue is replaced by an alanine (Hug and Sarre, 1993). However as these isoforms do show kinase activity (Liyanage et al., 1992; McGlynn et al., 1992; Selbie et al., 1993), the significance of this is unclear. A second consensus ATP binding motif is also present in the C4 domain of PKC α and β but not γ, though all the cPKCs have similar K_ms for ATP (Huang et al., 1988).

The C4 domain contains the phosphate transfer region and the putative substrate recognition site. The phosphate transfer region contains a triplet of amino acids (DFG) which represent the most highly conserved sequence in the catalytic domain of protein kinases (Kemp and Pearson, 1990), although PKC ζ varies by one amino acid in this sequence (Hug and Sarre, 1993). The aspartic acid residue of this triplet is thought to be involved in the base-catalysed transfer of the phosphate to the protein substrate (Taylor, 1989). The distance of 105-108 amino acids (113 in PKC γ) between the ATP site and the phosphate transfer region is also conserved (Hug and Sarre, 1993). The exact location of the substrate binding site has not been established but it has been proposed to contain a stretch of 29 amino acids in the C4 domain, and synthetic peptide analogues of this region are thought to activate PKC by competing with the pseudosubstrate domain (House et al., 1989).

The variable regions differ distinctly between isoforms but are highly conserved between species, suggesting that they may play a role in determining the properties and function of the individual isoforms (Huang, 1989). The V1 domain is elongated in the nPKCs relative to the cPKCs and it has been suggested that this region may have a modulatory effect on the conserved domains that are common to both classes of PKCs (Hug and Sarre, 1993). The V3 region forms the hinge between the regulatory and catalytic domains and contains the sites for cleavage by both calpain and trypsin (Huang and Huang, 1986; Schaap et al., 1990). This region in PKC α also contains a potential Ca^{2+} binding domain, as suggested by its homology to Ca^{2+}/calmodulin dependent protein kinases, but this is not present in either the β or γ isoforms so its role in determining the sensitivity of this isoform to Ca^{2+} is unclear (Huang, 1989).

Protein kinase C μ varies extensively in the regulatory domain when compared to the other PKC isoforms (Johannes et al., 1994). It contains two cysteine-rich regions but these are separated by 79 amino acids, in contrast to the 15 or 22 amino acids in the cPKC and n PKCs respectively, and this isoform is unable to bind phorbol esters. It also contains a putative signal peptide and transmembrane domain, suggesting a permanent membrane location, in contrast to the transient membrane association of the other PKCs. Thus it is likely that PKC μ has a distinctly different cellular role to the other members of the PKC family.

1.3 PROPERTIES OF PKC ISOFORMS

The individual PKC isoforms vary greatly in both their patterns of expression and their biochemical properties, which differ particularly between isoforms in different groups, although there are similarities between group members.

Activation by phospholipids

Individual isoforms have distinct phospholipid preferences, though all may be activated in the presence of phosphatidylserine (PS)(Nishizuka, 1992). For example, cardiolipin (CL) is able to replace PS as a cofactor for PKC α, β and γ, in the presence of DAG or PDBu, resulting in near maximal activity for all three isoforms (Huang et al., 1988). However the subtle differences in the affinity of DAG or PDBu binding between these isoforms, when determined in the presence of PS, were accentuated when this lipid was replaced by presence of CL (Huang et al., 1988). Phosphatidylinositol (PI) can substitute for PS as a cofactor in activation of PKC α, βI (Kochs et al., 1993a) and ε (Koide et al., 1992), while for PKC δ, maximum tetradecanoylphorbolacetate (TPA) -stimulated activity was over 2 fold greater with PI than PS as a cofactor (Mizuno et al., 1991). Interestingly, phosphatidylinositol 4,5 bisphosphate (PIP_2) is able to substitute for DAG as an activator for PKC α, βI (Kochs et al., 1993a) and γ (Lee and Bell, 1991) but not for PKC βII (Lee and Bell, 1991). It has however been suggested that this activation occurs by a different mechanism (Azzi et al., 1992) and at a distinct site from that to which DAG binds (Lee and Bell, 1991). Protein kinase C η, but not PKC α or δ, is specifically activated by cholesterol sulphate alone and the level of stimulation is greater than that occurring with PS and phorbol ester as activators (Ikuta et al., 1994).

Some PKC isoforms can also be activated by fatty acids, although the mechanism by which this occurs is poorly understood and there have been some conflicting results. Activation of PKC by fatty acids has been shown to be both dependent on (Sekiguchi et al., 1987) and independent of Ca^{2+} (Murakami et al., 1986) and DAG has been shown to have no effect on this activation (Sekiguchi et al., 1987). However another later study suggested that PKC α, β and γ are all activated synergistically by DAG and unsaturated

fatty acids, with a further increase in activity in the presence of PS (Shinomura et al., 1991). In this case, unsaturated fatty acids including arachidonic, oleic, linoleic and linolenic were found to be active while saturated fatty acids, such as palmitic and stearic acids, were inactive and, in the presence of fatty acid and PS, these isoforms were active at basal levels of Ca^{2+}. Protein kinase C ε has also been shown to be activated by arachidonic acid in both the presence and absence of PS (Koide et al., 1992) while PKC δ in the presence of diglyceride and PS was strongly inhibited by this fatty acid (Ogita, 1992). Lysophospholipids, in particular lysophosphatidylcholine (LysoPC), are also able to potentiate DAG activation of PKC *in vitro* (Shinomura et al., 1991) although the concentration is critical as, at higher concentrations, lysoPC inhibits the activity of PKC α, β and γ (Sasaki et al., 1993). LysoPC shows no effect in the absence of PS and DAG, and it has been suggested that lysoPC acts by increasing the affinity of PKC for PS (Sasaki et al., 1993). The activity of the nPKCs δ and ε, however, is not enhanced by lysoPC.

Protein kinase C ζ shows distinctly different activation characteristics from the other PKC isoforms, as it exhibits a low level of constitutive, activator-independent activity (Liyanage et al., 1992; McGlynn et al., 1992) and is not responsive to phorbol esters (McGlynn et al., 1992; Nakanishi and Exton, 1992). The activity of this isoform, when purified from kidney, has been shown to be stimulated by PS and cis-unsaturated fatty acids, although the signals that activate this isoform *in vivo* are unknown (Nakanishi and Exton, 1992). There is evidence that PKC ζ, but not cPKC, is strongly activated by phosphatidylinositol 3,4,5-triphosphate (PIP_3), which is produced by phosphatidylinositol 3-kinase (PI-3 kinase)(Nakanishi et al., 1993). This suggests that this isoform may be activated downstream of tyrosine kinases (Nakanishi et al., 1993), although in another study the

nPKCs ε and η were shown to be activated by PIP_3 (Toker et al., 1994) On the basis of sequence homology, particularly in the C1 domain, it has been suggested that PKC ι (and PKC λ, the mouse homologue (Akimoto et al., 1994)) may share similar properties with PKC ζ, (Selbie et al., 1993) and phorbol ester binding studies detected no specific binding to PKC λ (Akimoto et al., 1994). Expression of PKC μ has shown that, *in vitro,* this isoform does not efficiently bind nor is it activated by phorbol esters (Pfizenmaier et al., 1993). The phospholipid requirements of PKC μ have not yet been investigated.

Substrate specificity

The PKC isoforms have been shown to vary greatly in their substrate specificity when their activity is assayed *in vitro* (Hug and Sarre, 1993). In particular, it is clear that the Ca^{2+}-independent PKC isoforms are unable to efficiently phosphorylate histone (Liyanage et al., 1992; Schaap and Parker, 1990). This is thought to be due to interactions of the regulatory domain with the substrate binding region of the catalytic domain, as proteolytic cleavage of PKC ε resulted in a constitutively-active catalytic subunit that was readily able to phosphorylate this substrate (Schaap et al., 1990). Furthermore, a chimeric protein containing the regulatory subunit of PKC γ fused to the catalytic subunit of PKC ε also showed histone phosphorylation activity (Pears et al., 1991). More recent studies have shown that the pseudosubstrate region alone is capable of influencing the substrate specificity and co-factor dependence of PKC η, as deletion of this region generated a cofactor-independent enzyme with high histone kinase activity (Dekker et al., 1993).

Tissue and cellular distribution

Each isoform shows a distinct tissue distribution (Nishizuka, 1988; Wetsel et al., 1992). Within each class of PKCs, there is at least one isoform that is virtually ubiquitous, while the other class members show a more specific tissue distribution (Nishizuka, 1992). PKC α , β, δ, ε and ζ are all found in many tissues throughout the body, including brain, skin, lung, spleen and thymus (Nishizuka, 1988; Wetsel et al., 1992), while PKC γ is exclusively found in the nervous system (Shearman et al., 1987) and PKC θ is predominantly located in skeletal muscle (Osada et al., 1992). It has been suggested that the ubiquitously-distributed isoforms may perform functions that are essential for cell survival, while the isoforms with a tissue specific distribution may have distinct functions in specialised differentiated cells (Hug and Sarre, 1993). There are however notable deviations in the distribution of the generally ubiquitous isoforms, for example PKC α has not been detected in liver where PKC β is the major isoform (Rogue et al., 1990), and it has been suggested that these isoforms may substitute for each other in different tissues with respect to identical or similar function (Hug and Sarre, 1993).

The intracellular distribution of different isoforms within unstimulated cells also varies considerably. PKC α is predominantly cytosolic in unstimulated cells (Borner et al., 1992b) while PKC β and γ are predominantly membrane associated (Nishizuka, 1988; Strulovici et al., 1991). The cellular distribution of both PKC δ and ε seems to depend on the cell type. Both are found predominantly in the particulate fraction in rat 6 fibroblasts (Borner et al., 1992b) and renal mesangial cells (Huwiler et al., 1991; Huwiler et al., 1992) while PKC δ is cytosolic in human platelets (Grabarek et al., 1992) as is PKC ε in GH_4C_1 pituitary gland cells (Kiley et al., 1990). In most cell lines PKC ζ seems to be cytosolic (Borner et al.,

1992b, Huwiler et al., 1992), although in Hela cells it has been reported to occur predominantly in the particulate fraction (Ways et al., 1992). PKC η in several human cell lines has been detected exclusively in the nucleus (Grief et al., 1992), although another report found that this isoform in mouse and human keratinocytes was predominantly located in the cytoplasm around the nucleus but not in the nucleus itself (Chida et al., 1994). Expression of PKC θ in COS cells results in the majority of this isoform appearing in the particulate fraction in unstimulated cells (Osada et al., 1992). Thus it would appear that, for most isoforms, the cellular location is not only determined by the primary sequence of the protein but also the cell type in which it is expressed. The exception to this may be PKC μ, as this isoform has a potential transmembrane domain in the V1 region which may result in the mature protein being permanently membrane-bound (Johannes et al., 1994).

It may be only limited regions of the protein that are responsible for dictating the properties and distribution of particular isoforms, as suggested by the βI and βII isoforms. These proteins are both products of the β gene produced by alternative splicing of the mRNA at the 3' end (Coussens et al., 1986). The resulting proteins share identical conserved domains and only differ in the V5 region, at the carboxy terminus of the catalytic domain, the amino acids of this region showing 50% identity (Parker et al., 1989). However these two isoforms have been shown to vary in their activation characteristics, for example βII but not βI may be activated by PIP_2 in place of DAG (Lee and Bell, 1991). The ratios in which these isoforms are present in many tissues varies (Farago and Nishizuka, 1990) as does their cellular location, as shown in neuronal cells where βI is sometimes associated with

the plasma membrane while βII is often localized in the Golgi complex (Nishizuka, 1988).

The individual nature of the activation characteristics of each isoform as determined *in vitro* may reflect the ability of different isoforms to be activated in response to specific agonists.

1.4 MECHANISM OF PKC ACTIVATION

Activation of most of the PKC isoforms is dependent on PS and either DAG or phorbol esters, along with Ca^{2+} for the cPKCs (Huang, 1989). Diglycerides competitively inhibit phorbol ester binding so it is thought that both these types of activators interact with the same site (Kazanietz et al., 1993; Sharkey and Blumberg, 1985). The binding of PS is strongly co-operative when measured in a mixed micelle assay (Hannun et al., 1985). Binding of phospholipid cofactors and phorbol esters or DAG is thought to induce a conformational change that results in unmasking of the catalytic domain (Huang, 1989). Autoinhibitory domains have been identified for a number of protein kinases that act by interacting with elements of the catalytic domain (Soderling, 1990). A pseudosubstrate domain has been identified in the C1 region of PKC (House and Kemp, 1987) which resembles the natural substrate recognition sequence for PKC but lacks the serine or threonine residue for phosphorylation. It is suggested that this domain in the absence of cofactors binds to the active site maintaining the enzyme in an inactive state by preventing access to potential substrates (Fig 1.2). The interaction with cofactors is thought to cause a conformational change (Huang, 1989) resulting in the removal of the pseudosubstrate domain from the substrate binding site so allowing access to the substrate. This is supported by experiments which have shown that the pseudosubstrate domain is exposed to proteases when the enzyme binds

PS and DAG, but is masked when the enzyme is inactive (Orr et al., 1992). The substrate does however play a role in determining the cofactor requirement, at least *in vitro*, as there are some substrates, such as protamine sulphate, which are phosphorylated independently of phospholipid and DAG (Bazzi and Nelsestuen, 1987; Liyanage et al., 1992). It has however been suggested that a similar structural alteration occurs in the case of protamine suphate binding resulting in the exposure of the pseudosubstrate to proteolysis (Orr and Newton, 1994).

Translocation

Translocation of PKC from the cytosol to the membrane has been associated with PKC activation and also subsequent down-regulation. In the unstimulated cell, PKC is found in both the soluble and membrane fractions, the distribution between the two varying with PKC isoform content. However cytosolic PKC is recruited to the membrane in response to DAG and, in the cases of the cPKCs, increased levels of intracellular Ca^{2+}, and it has been suggested that the interaction of PKC with the membrane is stabilised when phospholipid and DAG or phorbol ester is bound (Bell, 1986). It has been proposed that there are two forms of membrane associated PKC, one that is reversibly bound and the other which is membrane inserted (Bazzi and Nelsestuen, 1988a). The latter form is constitutively active and shows no further increase in activity with either phorbol esters or Ca^{2+} (Bazzi and Nelsestuen, 1988b). Proteins that are thought to bind to PKC in its active form following translocation to the membrane have been identified in some cell types, for example hepatocytes (Mochly-Rosen et al., 1991a). These receptors for activated C kinase (RACKS) are suggested to play a role in anchoring PKC at the membrane, by direct protein-protein interaction (Mochly-Rosen et al., 1991b). They are

thought to bind PKC at a site distinct from the substrate binding site and they have been shown to increase PKC phosphorylation of other substrates. It has been suggested that they may prolong PKC activation by holding the enzyme molecule in the active conformation. A peptide that prevented PKC from binding to RACK 1 *in vitro* has been used to investigate the involvement of PKC interaction with RACKS in PKC-mediated function *in vivo* . Microinjection of this peptide into *Xenopus* oocytes was found to block insulin-induced oocyte maturation and PKC β translocation in response to insulin (Ron and Mochly-Rosen, 1994). The sequence of this peptide resembles that of an endogenous kinase C inhibitor protein (KCIP)(Toker et al., 1990), so modulation of PKC binding to RACKS may play a role in regulation of PKC activation *in vivo* (see Section 1.6). Other PKC binding proteins have also been identified that are also PS-binding proteins (Kiley and Jaken, 1994). These proteins are different from the previously mentioned RACKS as they are also PKC substrates, and while PKC binding to RACK 1 is irreversible *in vitro*, PKC binding to these proteins is decreased by phosphorylation.

Proteolysis and Downregulation

Proteolytic cleavage by calpain, a Ca^{2+}-dependent cysteine protease, occurs following activation and translocation of PKC, and the conformational change associated membrane binding causes the V3, hinge region of the enzyme to be more susceptible to proteolysis (Newton, 1993). This results in generation of the constitutively-active catalytic domain, called PKM, which is not regulated by DAG or phospholipid (Kishimoto et al., 1989). It is unclear, however, whether this is followed by further proteolysis and degradation, so terminating the kinase activity, or if the catalytic fragment, released from the membrane to the cytosol, persists as a

constitutively active kinase (Hug and Sarre, 1993). It has been suggested that PKM may play a role in long term PKC-mediated responses, for example long term potentiation (LTP)(Sacktor et al., 1993). Different isoforms vary in their susceptibility to such limited proteolysis (Huang et al., 1989) and a role for the V3 domain in determining the rate of cleavage has been suggested (Kishimoto et al., 1989).

Persistent stimulation of cells with either phorbol esters or agonists that activate PKC results in PKC down-regulation (Hug and Sarre, 1993), which is due to an increase rate of proteolysis (Young et al., 1987) and can occur without any change in the rate of synthesis. PKC isoforms are differentially downregulated *in vivo* (Hug and Sarre, 1993) and it has been suggested that this may be due to their different susceptibilities to proteolysis (Kiley et al., 1991). However, experiments where the V3 hinge domain of PKC α was exchanged for sequences from other membrane proteins that do not undergo proteolysis, showed that, while mutants varied in their rate of proteolysis by calpain, including one which was totally resistant to cleavage, all were sensitive to TPA-induced down-regulation (Junco et al., 1994). Selective down-regulation of certain PKC isoforms following agonist stimulation may be due to activation of these isoforms and not others by the stimulus, although the precise relationship between activation and down-regulation is unclear. The use of kinase-negative mutants to try and clarify this relationship has provided contradictory results. While there are some reports that kinase-negative mutants of PKC α and γ, when expressed in COS cells, could be downregulated by phorbol esters (Freisewinkel et al., 1991; Pears and Parker, 1991), an alternative report found that, in comparison to the wild type, there was severe impairment of phorbol ester-induced down-regulation of a kinase-negative mutant of PKC α, expressed in either COS cells or rat fibroblasts (Ohno et al., 1990). It has

been suggested that selective down-regulation of particular PKC isoforms may be at least partly due to the cellular compartment in which they are located (Kiley et al., 1991).

1.5 PKC ACTIVATION *IN VIVO*

Activation of protein kinase C *in vivo* occurs following hydrolysis of membrane phospholipids, as summarised in Figure 1.3. It was first detected in response to receptor-mediated inositol phospholipid breakdown, the resulting DAG increasing the affinity of PKC for Ca^{2+} and so activating it (Takai et al., 1979a). It has since been shown that DAG produced from phosphatidyl choline (PC) hydrolysis, by either a PC-specific phospholipase C (PLC) or by action of a phosphatase on the phosphatidic acid (PA) generated by phospholipase D (PLD), is also capable of activating PKC (Azzi et al., 1992), though different isoforms may be involved. This form of PLC may require tyrosine phosphorylation for its activation (Choudhury et al., 1991), which provides a potential route for cross-talk between cascades involving activation of tyrosine kinases and those involving PKC activation.

Receptor stimulation results in an initial peak of DAG production from breakdown of PI by PLC, with a simultaneous increase in intracellular levels of Ca^{2+} (Asaoka et al., 1992). This DAG is rapidly metabolised by DAG kinase but there is frequently a second peak of DAG production resulting from signal-induced PC hydrolysis (Exton, 1990). This second phase of DAG production can persist for several hours and may be essential for the prolonged activation of PKC necessary for long term cellular events, such as cell proliferation and differentiation (Azzi et al., 1992). It is possible that activation of PKC itself may be responsible for the sustained elevation of DAG (Nishizuka, 1992), as there is evidence to suggest that PKC may be involved in PLD activation (Billah and Anthes, 1990; Pelech and Vance,

1989). Phorbol esters or a membrane-permeant DAG activate PLD, although there is conflicting evidence concerning the requirement for ATP (Conricode et al., 1992; Olson et al., 1991; Tettenborn and Mueller, 1988) and the effect of kinase inhibitors on this process (Cao et al., 1990; Liscovitch and Amsterdam, 1989; Sandmann and Wurtmann, 1991; Yamada et al., 1991). It has been suggested that PKC stimulation of PLD may be independent of protein phosphorylation, although the mechanism by which this could occur remains unknown (Conricode et al., 1992).

Protein kinase C is also involved in the activation of phospholipase A_2 (PLA_2)(Godson et al., 1990; Hartung and Toyka, 1987), which occurs in response to most agonists that induce PI hydrolysis (Nishizuka, 1992). PLA_2 is responsible for breakdown of membrane phospholipids, in particular PC, to give free fatty acids, such as arachidonic acid, and lysophospholipid, both of which can either induce or enhance PKC activation (Shinomura et al., 1991), so providing a further mechanism for prolonged PKC activity (Nishizuka, 1992). As unsaturated fatty acids have been shown to lower the requirement of the cPKC for Ca^{2+} (Shinomura et al., 1991), this could enable these isoforms, activated by the initial DAG formation, which occurs concurrently with elevated levels of intracellular Ca^{2+}, to remain active once the Ca^{2+} levels have returned to basal. It is also possible that this production of unsaturated fatty acids as a later response may result in activation of isoforms that were not involved in the initial early response, for example PKC ζ which may be activated by free fatty acids but not by DAG (Nakanishi and Exton, 1992). As well as a role in potentiation of PKC stimulation by promoting further DAG production, PKC is also involved in the negative feedback control of this system, as PKC activation also causes down-regulation of agonist-induced PI hydrolysis (Pachter et al., 1992).

As previously mentioned PKC is also activated in response to growth factors, such as epidermal growth factor (EGF) and lysoPA (Ohno et al., 1994). Binding of these agonists to their receptors results not only in activation of PLC but also PI-3 kinase (Bjorge et al., 1990; Kumagai et al., 1993). This kinase is responsible for the phosphorylation of PI-4,5-P_2 to give PI-3,4,5-P_3 (PIP_3)(Whitman et al., 1988) The physiological roles of PIP_3 are not yet fully understood but it has been shown *in vitro* that this inositol phospholipid is capable of activating both the aPKCs and nPKCs (Nakanishi et al., 1993; Toker et al., 1994). It has also been suggested that activation of PKC by PI-3-kinase may be partly a direct phosphorylation event, as the catalytic subunit of this enzyme is thought to have protein kinase activity (S. Ohno; unpublished data).

1.6 Regulation of PKC activity

As previously mentioned, activation of PKC is associated with translocation to the membrane and subsequent limited proteolysis. It has been proposed that this may represent a physiological regulatory mechanism (Pontremoli et al., 1990) resulting in the termination of PKC activity. There are also a number of other mechanisms by which PKC activity may be regulated.

Phosphorylation

It has been suggested that phosphorylation may be important in the regulation of PKC. Many PKC isoforms, for example PKC δ and ε, are seen as doublets on western blots when obtained from native tissues, consistent the presence of different phosphorylation states (Koide et al., 1992; Ogita, 1992). Expression of PKC α in COS1 cells produced phosphorylated protein that was found to be inactivated by dephosphorylation and the initial phosphorylation event was shown to be independent of PKC activity (Pears

et al., 1992). The importance of phosphorylation in the regulation of PKC activity means that caution is necessary when expressing isoforms in non-mammalian expression systems as the correct phosphorylations may not occur. Problems have been encountered, for example, when using the baculovirus system to express PKC δ as initially only 1% of the protein produced was catalytically active, and inefficient phosphorylation was proposed as the reason for this problem (Rankl et al., 1994). The kinases responsible for PKC phosphorylation are not at present clearly defined. Protein kinase C δ, but not cPKCs, has been shown to be phosphorylated *in vitro* by the tyrosine kinases src and to a lesser extent fyn (Gschwendt et al., 1994), and overexpression of PKC δ, in both 32D hematopoietic cells and NIH-3T3 fibroblasts, resulted in tyrosine phosphorylation of this isoform in response to TPA (Li et al., 1994). In both these cases, tyrosine phosphorylation enhanced PKC δ activity, although tyrosine phosphorylated PKC δ, immunoprecipitated from keratinocytes expressing oncogenic ras, showed reduced activity when compared to nonphosphorylated PKC δ (Denning et al., 1993). Thus while it appears that PKC δ, but not some of the other PKC isoforms, is regulated by tyrosine phosphorylation, the effect on PKC δ activity seems to vary with cell type or the tyrosine kinase responsible.

Autophosphorylation

Autophosphorylation has been described for most protein kinases, although this is not necessarily linked to modulation of kinase activity (Miller and Kennedy, 1986). All isoforms of PKC have been shown to undergo autophosphorylation (Hug and Sarre, 1993) in an activator-dependent manner, though the K_m for ATP for this reaction is 10-fold lower that for substrate phosphorylation (Huang et al., 1986; Newton and Koshland,

1989). Both the regulatory and catalytic domain are autophosphorylated, modified residues being found in the amino-terminal region, the carboxy-terminal tail and the hinge section between the regulatory and catalytic domains, and this reaction is thought to follow an intramolecular mechanism (Flint et al., 1990; Huang et al., 1986). The sites of autophosphorylation are poorly conserved between PKC isoforms (Azzi et al., 1992), but some motif selectivity must exist because phosphorylated residues are often located next to other serine or threonine residues which show no autophosphorylation (Hug and Sarre, 1993). Different isoforms not only select different sites but they also vary in which type of amino acid is preferred. PKC α and ζ were found to autophosphorylate almost exclusively on serine residues, while PKC βI autophosphorylated primarily threonine residues and PKC βII showed no clear preference (S. Gaubatz and T Sarre, unpublished data from (Hug and Sarre, 1993)). Autophosphorylation of PKC results in increased affinity for phorbol esters, increased sensitivity to Ca^{2+} and increased rate of histone phosphorylation (Huang et al., 1986). It has also been suggested that autophosphorylation in the V3 region close to the site of calpain cleavage may change the susceptibility to proteolysis (Azzi et al., 1992).

Endogenous PKC inhibitors

The presence of endogenous inhibitors has been suggested as one mechanism by which PKC activity may be regulated *in vivo*. One group reported isolation of 3 closely related potent PKC inhibitor proteins from sheep brain, which were called kinase C inhibitor proteins (KCIP-1)(Toker et al., 1990). These acidic proteins of 29-33 kDa showed amino acid sequence homology with the carboxy-terminal region of the annexin family of Ca^{2+}/lipid binding proteins. They were subsequently identified as

members of the 14-3-3 family of proteins (Toker et al., 1992), which are found in most mammalian tissues and are thought to function as regulators in a variety of signal transduction pathways (Aitken et al., 1992). Inhibition of PKC by KCIP-1 does not appear to be due to competition with ATP, DAG, Ca^{2+} or substrate, and it has no effect on [^{3}H]PDBu binding (Toker et al., 1990). Part of the sequence of KCIP-1 resembles the carboxyl terminus of the annexins, a family of Ca^{2+} and lipid binding proteins, one of which (annexin V) has been shown to inhibit PKC (Schlaepfer et al., 1992). It has been suggested that the region in KCIP-1 that resembles the annexins may bind the regulatory domain of PKC, and a synthetic peptide based on this sequence, from annexin-I, has been shown to prevent PKC binding to RACKS (Mochly-Rosen et al., 1991b). It has therefore been suggested that these proteins may act as regulators of PKC translocation (Aitken et al., 1992).

Other endogenous PKC inhibitors have also been detected. A 17 kDa protein called protein kinase C inhibitor I (PKCI) was isolated from bovine brain and its amino acid composition was found to members of the resemble that of the calmodulin family of Ca^{2+}-binding proteins (McDonald et al., 1987). Its inhibitory effect on PKC, however, was reported not to involve competition with Ca^{2+} or phospholipid cofactors. The sequence published, however, was later found to correspond to an FK 506 binding protein that had co-purified with the PKC inhibitor (Mozier et al., 1991). Recently a 70 kDa protein from maize has been cloned and found to have high similarity to bovine PKCI (Simpson et al., 1994). While this 70 kDa protein has little effect on PKC activity alone, it has been shown that to synergise with KCIP (Robinson and Aitken, 1994). A peptide inhibitor (designated IN) has been detected, in phytohaemogglutinin-activated peripheral blood mononuclear cells, which is thought to act on the

regulatory domain of PKC as it was ineffective at inhibiting the catalytic domain generated by trypsin cleavage of PKC (Gandini et al., 1993). Therefore it appears that PKC activity may be regulated *in vivo* by a variety of endogenous inhibitors in different cell types.

Regulation of PKC gene expression

At present little is known about the factors responsible for the regulation of the PKC genes or their promoter sequences of the PKC genes (Hug and Sarre, 1993). Comments can only be made concerning the rat PKC γ and human PKC β promoters, as these are currently the only ones that have been cloned (Chen et al., 1990; Niino et al., 1992; Obeid et al., 1992). Both these promoters contain an AP1 site and at least one AP2 site. As these sites are responsible for conferring inducibility by phorbol esters on promoters containing them, it is possible that PKC β and γ are positively regulated by themselves or other PKC isoforms (Hug and Sarre, 1993). This could represent a means by which PKC mRNAs are replenished following PKC activation and subsequent degradation (Hug and Sarre, 1993). The PKC γ promoter contains a cAMP response element (Locker and Buzard, 1990), potentially allowing activation of the cAMP second messenger system to upregulate PKC-mediated responses. Deletion analysis of the PKC β promoter identified both positive and negative regulatory regions, and control of transcription by these regions is cell type specific (Niino et al., 1992). In order to identify the elements responsible for the different expression patterns shown by PKC isoforms, it will be necessary to compare the promoter sequences of these isoforms with those not yet isolated.

1.7 Cellular Roles of PKC

A wide variety of possible roles for PKC in cellular responses have been proposed, as shown in Table 1, as well as in crosstalk between different second messenger pathways (Houslay, 1991). In the nervous system, PKC has been shown to be involved in the enhancement of neurotransmitter release (Pozzan et al., 1984; Tanaka et al., 1984), regulation of Na^+ (West et al., 1991) and Ca^{2+} ion channels (Campbell et al., 1988) and modulation of neuronal plasticity (Malenka et al., 1986; Wang and Feng, 1992). Protein kinase C participates in inflammatory processes and immune responses, as signaling through PKC has been implicated in the activation of neutrophils (Tauber, 1987), lymphocytes (Berry et al., 1990; Cambier and Ransom, 1987) and differentiation of myeloid cells to macrophages (Aihara et al., 1991). There is also good evidence that PKC plays an important role in the activation of platelets (Rink et al., 1983; Wang et al., 1993).

Protein kinase C is involved in nuclear events, such as control of the cell cycle and regulation of gene expression (Nishizuka, 1992). The Ca^{2+}-independent PKCs may be activated as part of the cascade initiated by growth factors such as EGF, leading to regulation of the cell cycle (Hattori et al., 1992; Rossomando et al., 1992). Protein kinase C has been shown to phosphorylate DNA topoisomerase (Pommier et al., 1990) and lamin B (Fields et al., 1988); nuclear proteins both involved in the control of DNA synthesis. PKC is involved in regulation of gene expression, as treatment of cells with phorbol esters results in the increased transcription of a number of genes, due to activation of transcription factors. Activation of PKC results in phosphorylation of the inactive I-κB/NFκB complex on the inhibitory protein (IκB)(Ghosh and Baltimore, 1990), releasing the NFκB transcription factor which then moves to the nucleus and binds specifically to DNA. PKC also

activates the AP1 transcription factor, a complex of the *c-fos* and *c-jun* proteins, which confers inducibility by phorbol esters to genes containing the TPA-response element in their promoter (Karin, 1992). The activation of this complex by PKC seems to involve pathways leading to the both the phosphorylation of c-*fos* and the dephosphorylation of c-*jun* (Karin, 1992). Modulation of gene expression by PKC is thought to occur not only at the transcriptional level but also by regulation of the rate of translation (Hug and Sarre, 1993). The ribosomal S6 protein, as well as the initiation factor complex, have been shown to be phosphorylated in response to phorbol esters (Morley and Traugh, 1990). This protein is thought to be phosphorylated predominantly by ribosomal S6 kinases and PKC has been implicated in one of the two phases of EGF-induced activation of S6 kinase in Swiss 3T3 cells (Susa et al., 1989). Components of the translation initiation complex have also been found to be phosphorylated both *in vitro* and in cultured cells by PKC (Morley et al., 1991; Smith et al., 1991). It has been suggested that translational control mechanisms may be involved in PKC-mediated proliferation and differentiation events (Hug and Sarre, 1993), as these events are accompanied by significant alterations in the rate of protein biosynthesis (Hershey, 1991). Indeed, loss of the PKC-dependent phase of S6 kinase activation resulted in a diminished mitogenic response to EGF in Swiss 3T3 cells (Susa et al., 1989).

In addition to these stimulatory events, PKC also plays a role in the negative feedback regulation of a variety of cell signalling processes, involved in both the short term and prolonged responses of the cell. In the short term, PKC appears to be involved in decreasing intracellular Ca^{2+} levels elevated by IP_3 (Nishizuka, 1988). It has already been mentioned that PKC is involved in inhibiting Ca^{2+} mobilisation by blocking receptor-mediated hydrolysis of PI, but it is also thought to stimulate IP_3 hydrolysis by

activating an IP_3 phosphatase (Persons et al., 1988). It is proposed that PKC may stimulate the removal of intracellular Ca^{2+} by activation of the Ca^{2+}-transport ATPase and the Na^{+}/Ca^{2+} exchange protein (Nishizuka, 1986).

Protein kinase C also negatively regulates long-term responses such as cell proliferation (Nishizuka, 1988). The EGF receptor has been shown to be phosphorylated by PKC both *in vitro* and *in vivo*, resulting in down-regulation by decreasing high-affinity binding of EGF and inhibiting ligand-induced tyrosine phosphorylation (Schlessinger, 1986). A similar response occurs in T-lymphocytes; treatment of these cells with TPA and a Ca^{2+} ionophore resulting in phosphorylation of the T-cell receptor and preventing any further proliferative response to antigen (Cantrell et al., 1985).

Many of the effects seen following PKC activation are not a result of direct phosphorylation of the effector proteins but due to PKC initiating cascades of phosphorylation by activation of other kinases. Activation of mitogen activated protein kinase (MAP kinase) has been shown following PKC activation (Adams and Parker, 1992) although the classical cascade of MAP kinase activation is through ras, raf and MAP kinase kinase (MEK)(Marx, 1993). It has been shown that Raf-1 may by activated by serine phosphorylation and PKC has been suggested as a likely candidate (Carroll and Stratford-May, 1994). Recent studies have shown that PKC can phosphorylate and stimulate Raf-1 *in vitro* (Kolch et al., 1993), and there is evidence that direct phosphorylation and activation of Raf-1 by PKC occurs *in vivo* in hematopoietic cells (Carroll and Stratford-May, 1994). Activation of MAP kinase provides an alternative, indirect means by which PKC can modulate gene transcription, as MAP kinase is also able to phosphorylate transcription factors resulting in enhancement of gene expression (Edwards, 1994).

Protein kinase C also plays a role in cross talk between a number of second messenger systems. As well as the previously mentioned effects on the MAP kinase cascade, PKC may also be involved in the regulation of cAMP-mediated intracellular signalling pathways. Phorbol esters have been shown to both stimulate and inhibit cAMP levels in a number of cell types, tissues and cell lines (Houslay, 1991). These diverse responses may be due to the actions of different PKC isoforms as well components of the cAMP cascade, such as heterotrimeric G-proteins, adenylyl cyclase, and phosphodiesterase. In the HT4 neural cell line, phorbol ester stimulation of cAMP levels was found to synergise with adrenaline receptor-mediated effects, although some activation of the cAMP cascade was found to be essential for phorbol-induced stimulation. It was therefore proposed that PKC was only able to phosphorylate a component of the adenylate cyclase activation mechanism when in an activated not resting state (Morimoto and Koshland, 1994).

There is also evidence that PKC can influence the cGMP/guanylate cyclase system. Protein kinase C is able to phosphorylate guanylate cyclase *in vitro*, leading to its activation, (Zwiller et al., 1985) and treatment of pinealocytes with phorbol esters results in increased cGMP levels (Ho et al., 1987). Recent studies on rat colonic particulate guanylate cyclase have shown that activation of this enzyme by both raised intracellular Ca^{2+} levels and the vitamin D3 metabolite, 1,25-dihydroxycholecalciferol, occur via a PKC-dependent pathway (Khare et al., 1993; Khare et al., 1994). In PC12 cells, PKC has been shown to phosphorylate and activate soluble guanylate cyclase (Louis et al., 1993), so providing one mechanism whereby agonist-stimulation of PI hydrolysis can result in raised levels of cGMP.

1.8 ROLES OF SPECIFIC PKC ISOFORMS

The number of PKC isoforms, their variations in biochemical properties and expression in different cell types has led to the suggestion that different isoforms may activate different cellular pathways and phosphorylate different proteins (Azzi et al., 1992). This is supported by studies showing that the expression of PKC isoforms in haemopoietic cells is cell type and B cell differentiation stage-specific (Mischak et al., 1991), while the expression of PKC isoforms is modulated during granulocyte differentiation (Hashimoto et al., 1990). Furthermore the exclusive localisation of PKC γ in the central nervous system (Shearman et al., 1987) suggests that this isoform has a specialised function (Nishizuka, 1988). Roles have been proposed for different PKC isoforms on the basis of which isoforms are activated, as reflected by translocation, on stimulation of cells with different agonists (Wilkinson and Hallam, 1994), as well as the selective down-regulation of isoforms on prolonged agonist stimulation. The possible roles of isoforms in fibroblast transformation by the *ras* oncogene has been studied by measuring the levels of isoform expression, which showed PKC α and δ to be increased but PKC ε decreased, as detected both at the mRNA and protein level (Borner et al., 1992a).

Studies of the effects of overexpression of individual isoforms have been widely used to evaluate the roles of different PKC isoforms. PKC δ has been implicated in control of the cell cycle, as when this isoform, but not PKC α, βII or ζ, is overexpressed in CHO cells, treatment with phorbol esters prevents progression of the cell cycle through M phase (Watanabe et al., 1992). PKC δ has also been suggested to play a role in MAP kinase activation as cells overexpressing this isoform showed prolonged phorbol ester-induced stimulation of MAP kinase activity (Yamaguchi, Ogita and Nishizuka, unpublished data from Nakamura and Nishizuka, 1994). Other

isoforms are also likely to play a role in MAP kinase activation through Raf-1, however, as PKC α has been shown to be able to phosphorylate and activate Raf-1 *in vitro* (Kolch et al., 1993). Overexpression of PKC isoforms, along with a reporter construct under the control of a TPA response element, has provided evidence for the involvement of PKC δ, ε and ζ but not α in serum, EGF and lysoPA-induced stimulation of gene expression (Ohno et al., 1994). This is consistent with the observation that EGF and lyso PA cause an increase in phosphorylation levels of PKC δ and ε but not α (Ohno et al., 1994). There has been considerable interest in elucidating the isoforms involved in cell proliferation, due to the implications for cancer therapy. Overexpression of PKC α does not appear to result in cell transformation (Borner et al., 1991; Eldar et al., 1990) while PKC δ slowed the growth rate of NIH 3T3 cells, PKC ε having the opposite effect on these cells (Hug and Sarre, 1993). Contradictory results have been obtained for PKC βI and PKC γ, PKC βI causing transformation of rat 6 fibroblasts (Housey et al., 1988) but not of rat liver epithelial cells (Hsieh et al., 1989), while PKC γ may (Persons et al., 1988) or may not (Cuadrado et al., 1990) cause transformation of NIH 3T3 cells. It therefore appears that the effects of overexpression may depend on the cell line in which it is expressed, as well as on the levels of expression that result (Hug and Sarre, 1993). It has also been suggested that overexpression may result in incorrect signalling, through the phosphorylation of non-physiological substrates, as the overexpressed protein may undergo inappropriate compartmentalisation (Wilkinson and Hallam, 1994).

Dominant kinase-negative mutants have also been used to evaluate the roles of different isoforms. By this method, PKC ζ has been implicated in mitogenic signal transduction and placed downstream of ras activation (Berra et al., 1993). This isoform has also been linked to activation of the

transcription factor NFκB (Diaz-Meco et al., 1993). A further role proposed for this isoform is in the maintenance of LTP, as PKC ζ may show persistent activation by conversion to the constitutively-active PKM following induction of LTP (Sacktor et al., 1993).

The number of roles that have been proposed for different PKC isoforms, along with the sometimes contradictory results obtained when trying to ascribe roles to particular isoforms, suggests that the selection of isoforms activated in response to a particular stimulus may be determined not only by the biochemical properties of each isoform, but also by the type of cell, the isoforms that it expresses and the subcellular distribution of those isoforms.

1.9 PKC PHARMACOLOGY

Protein kinase C has been implicated in many cell responses by the use of PKC activators to mimic agonist-induced responses or by using PKC inhibitors to block the pathway (Wilkinson and Hallam, 1994). The most commonly used PKC activators, in addition to diglycerides, are the phorbol esters and related compounds, including phorbol 12,13-dibutyrate (PDBu) and TPA. However while the diacylglycerol, 1-oleoyl-2-acetylglycerol has similar affinities for all the PKC isoforms (Kazanietz et al., 1993), some of the phorbol esters show selectivity between isoforms (Kazanietz et al., 1993; Ryves et al., 1991). Phorbol esters like PDBu and 12-deoxyphorbol esters show a slight preference for the Ca^{2+}-dependent isoforms (Kazanietz et al., 1993), while sapintoxin A will activate PKC α, βI, γ and ε but not PKC δ. The mezerein-like compounds are also more potent at activating the cPKCs, the most striking of which, thymeleatoxin, shows almost 20-fold less affinity for PKC ε and η over PKC βI (Kazanietz et al., 1993). Another commonly used phorbol ester is TPA, which is effective at activating the Ca^{2+}-dependent

PKC isoforms, particularly PKC βI, in the absence of Ca^{2+} (Ryves et al., 1991). Phorbol esters and other diterpenes are not the only pharmacological agents that may be used as PKC activators. The macrocyclic lactone, bryostatin, is also a potent PKC activator which is active at picomolar concentrations rather than the nanomolar concentrations required for the phorbol esters, although it also acts by binding to the regulatory domain (Blumberg and Pettit, 1992).

Protein kinase C activators have been shown to vary in their tumour-promoting capabilities. The phorbol esters, such as TPA, act as full tumour promoters, producing inflammation and induce ornithine decarboxylase activity, as well as decreasing epidermal differentiation (Slaga et al., 1980). Diterpenes, such as mezerein, induce some of the biological effects of the phorbol esters, for example induction of cell proliferation and stimulation of DNA synthesis, but are up to 50 times less active than TPA as complete tumour promoters, although they are potent second stage tumour promoters (Slaga et al., 1980). As these differences in biological activity may reflect the involvement of different PKC isoforms, the development of isoform-specific activators would prove useful for evaluating the roles of particular PKC isoforms *in vivo*.

The use of phorbol esters to mimic agonist-induced responses thought to involve PKC must, however, be treated with some caution. Phorbol esters, unlike DAG, are metabolically stable and the use of high concentrations may result in a decrease in the substrate specificity of PKC phosphorylation *in vivo* (Wilkinson and Hallam, 1994). Many phorbol esters, unlike DAG, are also able to activate Ca^{2+}-dependent PKC isoforms in the absence of Ca^{2+} (Ryves et al., 1991), so the responses initiated by these compounds may not entirely resemble those occurring physiologically following agonist stimulation. Furthermore PKC is not the only cellular

receptor for phorbol esters. N-chimaerin is a $p21^{rac}$-GTPase-activating protein (rac-GAP) as well as a high affinity phorbol ester receptor and the rac-GAP activity of n-chimaerin has been found to be stimulated by PS alone or synergistically with phorbol esters (Ahmed et al., 1993). Another high affinity phorbol ester receptor is unc-13, a protein identified in *Caenorhabditis elgans* that plays a role in synaptic transmission (Maruyama and Brenner, 1991). This protein shows sequence identity to the C1/C2 domains of PKC and also binds phorbol esters in a phospholipid dependent manner (Ahmed et al., 1992).

Protein kinase C inhibitors may be divided into two categories, depending on whether they bind to the regulatory or catalytic domain. Some regulatory domain inhibitors interfere with Ca^{2+} and phospholipid binding, for example dibucaine, (Mori et al., 1980) but these are not specific for PKC as they also inhibit Ca^{2+}/calmodulin dependent kinases (Hidaka and Hagiwara, 1987). More specific inhibitors act by inhibiting phorbol binding, for example gossypol and the fungally-derived calphostins. The most potent calphostin is calphostin C, which shows a similar potency for PKC α, β and γ. It requires photoactivation in order to inhibit phorbol binding, although the mechanism of this light-dependent inhibition is not fully understood (Azzi et al., 1992). Tamoxifen and adriamycin are also regulatory domain inhibitors that are thought to interfere with the interaction between PKC and phospholipids (Su et al., 1985; Wise and Kuo, 1983), although both of these compounds and also gossypol are also able to inhibit the catalytic fragment of PKC, with only a 2-3 fold difference in potency. It has been suggested that many PKC inhibitors may have multiple sites of action (Nakadate et al., 1988). The problem with the regulatory domain inhibitors is that they tend to be hydrophobic and so may exert non-specific effects on kinases and at the cell membrane. Sphingosine is a

physiological inhibitor of PKC and it has also been used to inhibit PKC *in vitro*, although it is not specific for PKC as it also inhibits calmodulin-dependent protein kinases (Jefferson and Schulman, 1988). It is another inhibitor which is thought to have multiple sites of action, as it is reported to inhibit [^{3}H] PDBu binding at concentrations similar to those at which it inhibits kinase activity, but it is also capable, at low concentrations of phospholipid, to inhibit the activity of the catalytic fragment (Nakadate et al., 1988). Sphingosine has, however, been shown to have mitogenic effects that are independent of PKC (Zhang et al., 1990) and it is readily converted to sphingosine 1-phosphate (Zhang et al., 1991), which also has a variety of cellular effects including activation of PLD (Spiegel, 1993) and release of Ca^{2+} independent of IP_3, (Ghosh et al., 1990). Thus sphingosine is not a useful tool for evaluation of PKC activation *in vivo*.

There is also a variety of PKC inhibitors that act on the catalytic domain. These include the isoquinolines, such as H7, and the microbially-derived indole carbazoles, including staurosporine and K252a. These are all thought to interact with the ATP binding site (Hidaka et al., 1984; Tamaoki et al., 1986), although their precise sites of action seem to vary. H7 competes kinetically with ATP (Hidaka et al., 1984) but only partially protects the ATP binding site from covalent chemical reagents (Ohta et al., 1988) and it displaces [^{3}H]*N,N*-dimethyl staurosporine binding only at very high concentrations (Thomson et al., 1991). Staurosporine has a high potency in cells that appears to be incompatible with a solely ATP-competitive mechanism (Rüegg and Burgess, 1989) and it has been reported that the inhibitory effect of this compound is not influenced by the presence of excess ATP (Tamaoki et al., 1986). Thus the exact mechanism of inhibition by staurosporine is unclear. The problem with these three inhibitors is that they all show poor selectivity for PKC over other kinases, as the ATP binding

region of PKC is highly homologous with that of other serine/threonine kinases as well as tyrosine kinases (Hanks et al., 1988). Recently the bisindolylmaleimide inhibitors, including Ro31-8220 and GF109203X, have been developed, based on the structure of staurosporine. These compounds have also been shown to be competitive inhibitors with respect to ATP, but display high selectivity for PKC over other kinases (Davis et al., 1989; Toullec et al., 1991) and are reported to vary in their potency on different PKC isoforms (Wilkinson et al., 1993). Another related compound, Go 6976, is also a potent, selective PKC inhibitor which has been shown to distinguish between the Ca^{2+}-dependent and Ca^{2+}-independent PKC isoforms, as it inhibited PKC α and βI at nanomolar concentrations but even micromolar concentrations had no effect on PKC δ, ε or ζ (Martiny-Baron et al., 1993). Recently another ATP competitive inhibitor, rottlerin, has been identified with specificity for PKC δ over other PKC isoforms, which are at least one order of magnitude less sensitive to this inhibitor (Gschwendt et al., 1994). While rottlerin showed some specificity for PKC δ over other protein kinases, it inhibited Ca^{2+}/calmodulin-dependent protein kinase III with similar potency to PKC δ. It is possible that further development of rotterlin- or staurosporine-related compounds may result in inhibitors with improved isoform-selectivity which would be invaluable tools for dissecting the physiological roles of individual isoforms.

The substrate binding site provides an alternative target for catalytic domain inhibitors, the most effective of which are based on the PKC pseudosubstrate region. As previously mentioned, this region is an autoinhibitory domain that is thought to interact with the substrate binding site, in the absence of activators, so preventing PKC activity. It has been shown that synthetic peptides consisting of this sequence are PKC substrate antagonists, due to the presence of the basic residue determinants

necessary for substrate recognition. These peptides were found to be able to inhibit both substrate phosphorylation and autophosphorylation, and were specific for PKC over other kinases that require arginine in their sustrate recogniton motif, such as cAMP-dependent protein kinase (House and Kemp, 1987). In view of the variation between PKC isoforms in their pseudosubstrate domain, it is possible that these peptides may show some selectivity between PKC isoforms. This is supported by evidence that in *Xenopus* oocytes, which contain PKC α, β, γ and ζ, TPA-evoked PKC activity was inhibited by the α but not the ζ pseudosubstrate inhibitor, while immunoprecipitated PKC ζ was inhibited by the ζ but not α pseudosubstrate inhibitor (Dominguez et al., 1992).

1.10 PKC IN THE ANTERIOR PITUITARY GLAND

As previously mentioned, even within one tissue type, different activators and inhibitors vary in their ability to affect individual PKC-mediated responses. This may be due to these pharmacological agents affecting only some of the PKC isoforms present in that tissue. In the anterior pituitary gland, there is increasing evidence for the existence of pharmacologically distinct forms of PKC and their involvement in a number of anterior pituitary cell responses. The PKC activators mezerein and PDBu have been shown to induce release of both growth hormone (GH) and luteinising hormone (LH) from rat anterior pituitary tissue, but these responses vary in their sensitivity to PKC inhibitors (Thomson et al., 1993b). Both responses are blocked by staurosporine and the more selective PKC inhibitor Ro31-8220. However inhibition of LH release by H7 shows two components, one H7-sensitive and one relatively H7 resistant, while GH release is totally resistant to this inhibitor. Agonist-induced hormone release can also show this unusual pharmacological profile. Luteinising

hormone-releasing hormone (LHRH) is able to increase the responsiveness of gonadotrophs, the LH-secreting cells in the anterior pituitary, to LHRH. This process, known as self-priming, is also mediated by PKC, as it is blocked by Ro31-8220 and other PKC inhibitors, but the PKC involved is relatively resistant to inhibition by H7 (Johnson et al., 1992b).

Protein kinase C is also involved in the modulation of L-type Ca^{2+} channels in anterior pituitary tissue, and it has been shown that this is involved in PDBu-induced release of GH but not LH (Johnson et al., 1993). PDBu has been shown to have dual effects on these channels depending on cell type, as, in pituitary tissue, K^+-induced Ca^{2+} influx is facilitated by PDBu, while in the clonal pituitary GH_3 cell line, PDBu inhibits this response (MacEwan and Mitchell, 1991). There is evidence to suggest that the facilitation in pituitary tissue is mediated by the H7-resistant form of PKC mentioned above, while the inhibition seen in GH_3 cells is the result of a different, H7-sensitive form of PKC (MacEwan et al., 1991; MacEwan et al., 1992).

Studies have been conducted to identify the downstream targets of this H7-resistant PKC-like kinase. It has been shown to be involved in the activation of mitogen-activated protein kinase (MAP kinase) by LHRH, in both pro-oestrous anterior pituitary tissue and the αT3-1 gonadotroph cell line (Mitchell et al., 1994; Sim and Mitchell, 1994). This H7-resistant PKC-like kinase has also been implicated in the activation of PLA_2 induced by both PDBu and LHRH; LHRH-induced arachidonate production is sensitive to Ro31-8220 but relatively resistant to H7, while the PDBu-induced response shows two components of varying sensitivities to H7 (Thomson and Mitchell, 1993). It is possible that this PLA_2 response may be downstream of MAP kinase, as the high molecular weight form of PLA_2 has a potential phosphorylation site for MAP kinase (Lin et al., 1993). This is

supported by evidence that the TPA-evoked activation of PLA_2 in macrophages is a result of PKC activation of MAP kinase (Qui and Leslie, 1994) and a mechanism for agonist-induced activation of $cPLA_2$ involving PKC activation of MAP kinase has been proposed from overexpression studies of $cPLA_2$ in COS and CHO cells (Lin et al., 1993).

1.11 THE AIM OF THIS STUDY

PKC activity showing a similar pharmacological profile to that described above in these anterior pituitary cell events (that is induced by phorbol esters and inhibited by staurosporine but not H7) has been detected in the cytosolic fraction from anterior pituitary but not that obtained from midbrain, in an *in vitro* kinase assay. The aim of this study has been to further characterise this H7-insensitive PKC-like kinase, both in terms of its pharmacology, biochemical properties and tissue distribution, and by further purification and molecular biological approaches, to explore the possibility that this may represent a novel isoform of PKC or an unknown PKC-like kinase.

Table 1 Possible roles of PKC in cellular responses

Roles that have been proposed for PKC *in vivo* in a variety of tissues and cell types are indicated. Adapted from (Nishizuka, 1986).

Table 1.1

Tissues and cells	Responses
Endocrine systems	
Adrenal medulla	Catecholamine secretion
Adrenal cortex	Aldosterone secretion
Pancreatic islets	Insulin release
Pituitary cells	Pituitary hormone release
Exocrine systems	
Pancreas	Amylase secretion
Gastric gland	Pepsinogen secretion
	Gastric acid secretion
Alveolar gland	Surfactant secretion
Nervous system	
Neuromuscular junction	Transmitter release
Caudate nucleus	Acetylcholine release
PC12 cells	Dopamine release
Neurones	Dopamine release
Muscular systems	
Vascular smooth muscle	Muscle contration
	Muscle relaxation
Inflammation and immune systems	
Platelets	Serotonin release
	Arachidonate release
Neutrophils	Superoxide generation
	Lysosomal enzyme release
Basophils	Histamine release
Mast cells	Histamine release
Lymphocytes	T-lymphocyte activation
	B-lymphocyte activation
Metabolic and other systems	
Adipocytes	Lipogenesis
	Glucose transport
Hepatocytes	Glycogenolysis

Figure 1.1 Structure of the PKC isoforms.

A schematic representation of the cDNA sequences of the known PKC isoforms. The cPKCs are Ca^{2+}-dependent while the nPKCs and aPKCs are Ca^{2+}-independent. The aPKCs unlike the other isoforms are not dependent on phorbol esters. The boxed regions represent the conserved domains (C1-4) whilst V1-5 are the variable domains. Cys, cysteine-rich regions; P, pseudosubstrate domain. This figure is adapted from (Nishizuka, 1992).

Figure 1.1

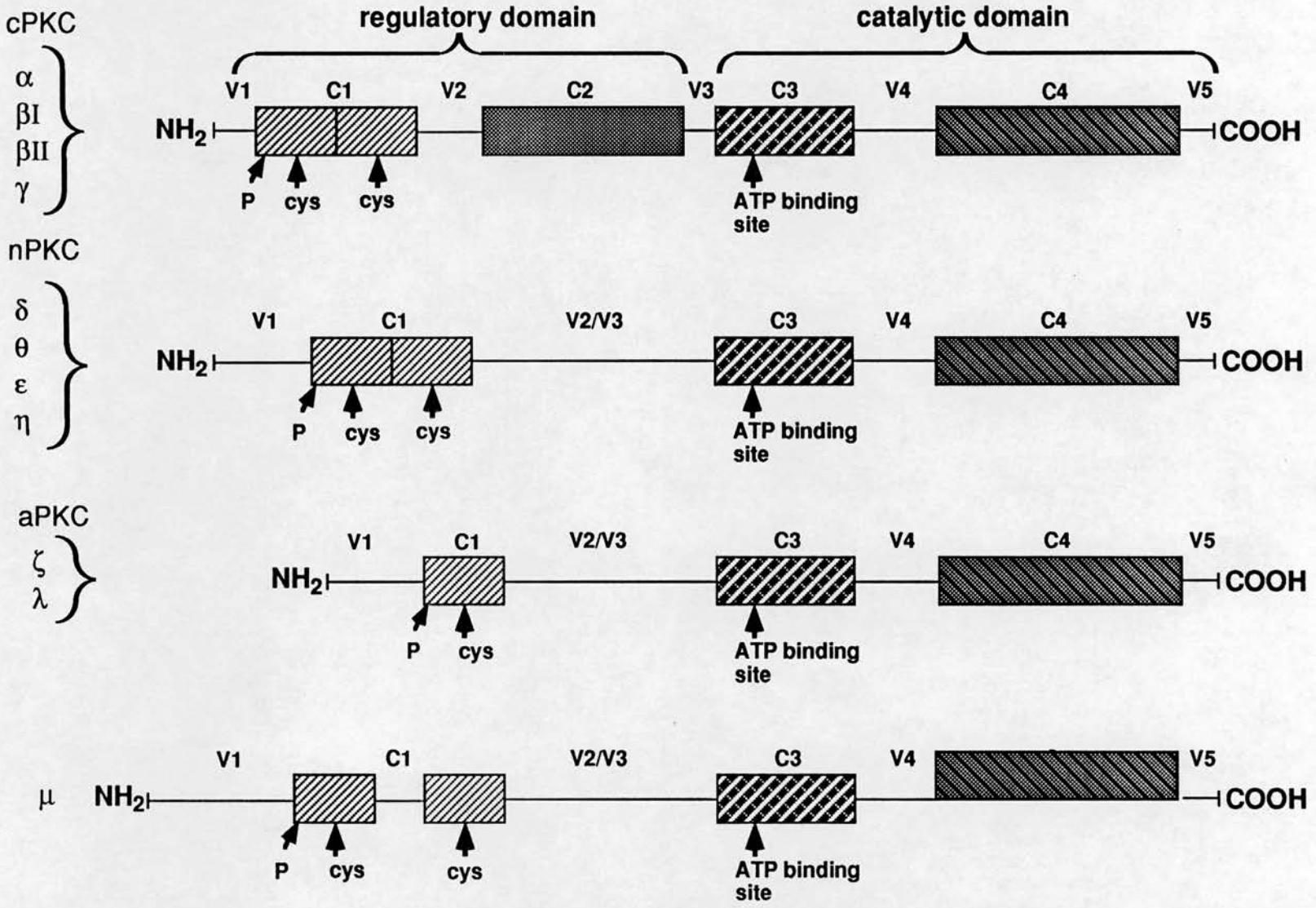

cPKC
α
βI
βII
γ
regulatory domain
catalytic domain
V1
C1
V2
C2
V3
C3
V4
C4
V5
NH2
COOH
P
cys
cys
ATP binding site
nPKC
δ
θ
ε
η
V1
C1
V2/V3
C3
V4
C4
V5
NH2
COOH
P
cys
cys
ATP binding site
aPKC
ζ
λ
V1
C1
V2/V3
C3
V4
C4
V5
NH2
COOH
P
cys
ATP binding site
μ
V1
C1
V2/V3
C3
V4
V5
NH2
COOH
P
cys
cys
ATP binding site

Figure 1.2 Proposed mechanism of PKC activation.

In the absence of DAG and phospholipid (and Ca^{2+} for the cPKCs), the pseudosubstrate region of the regulatory domain interacts with the substrate binding site so preventing access to potential substrates. Binding of DAG and phospholipid results in a conformational change which removes the pseudosubstrate domain allowing the substrate binding domain to interact with substrate proteins. Cleavage of the hinge region by proteases removes the regulatory domains resulting in a constitutively active catalytic domain. R regulatory domain, C catalytic domain, S substrate binding site, P pseudosubstrate domain.

Figure 1.2

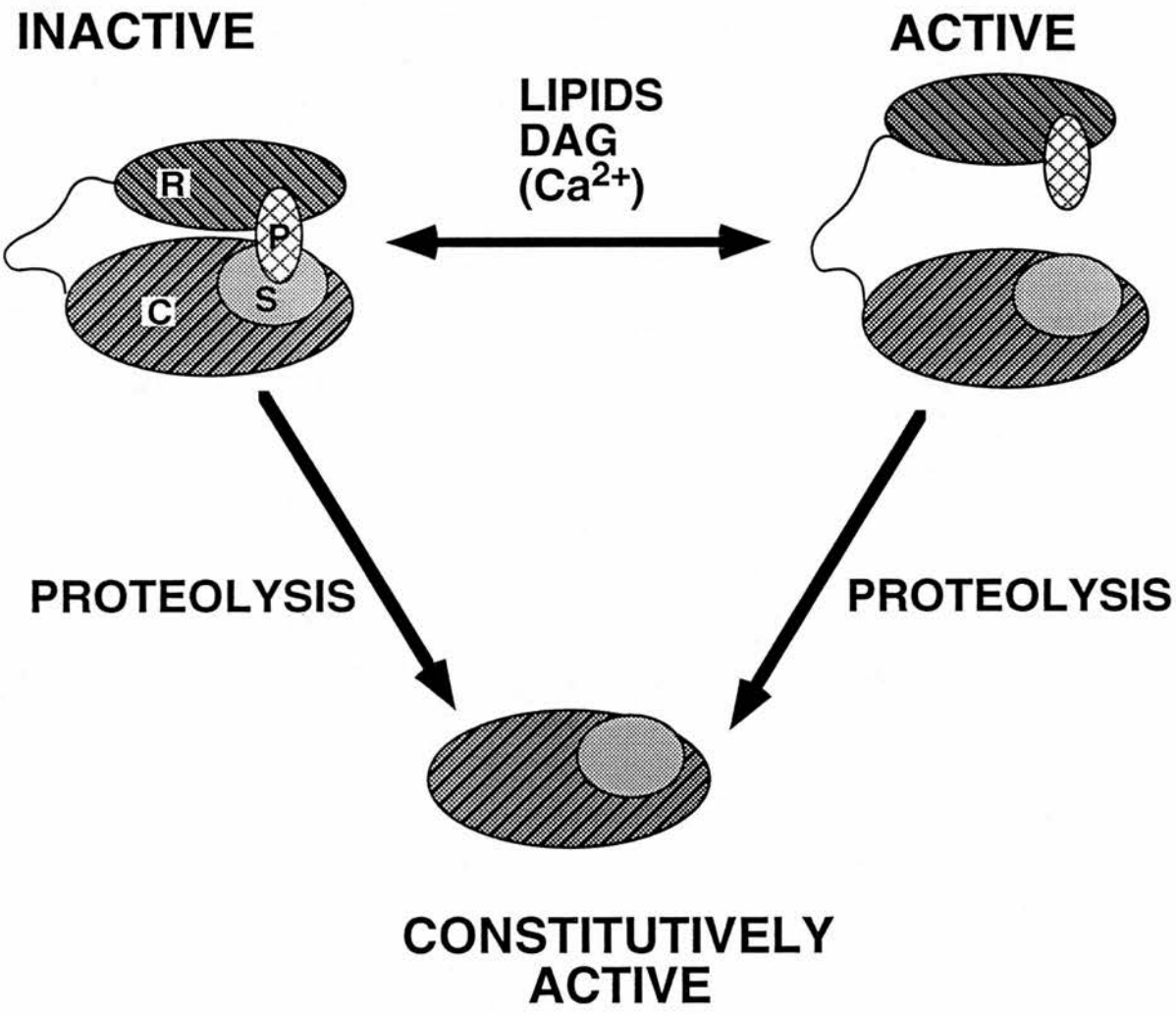
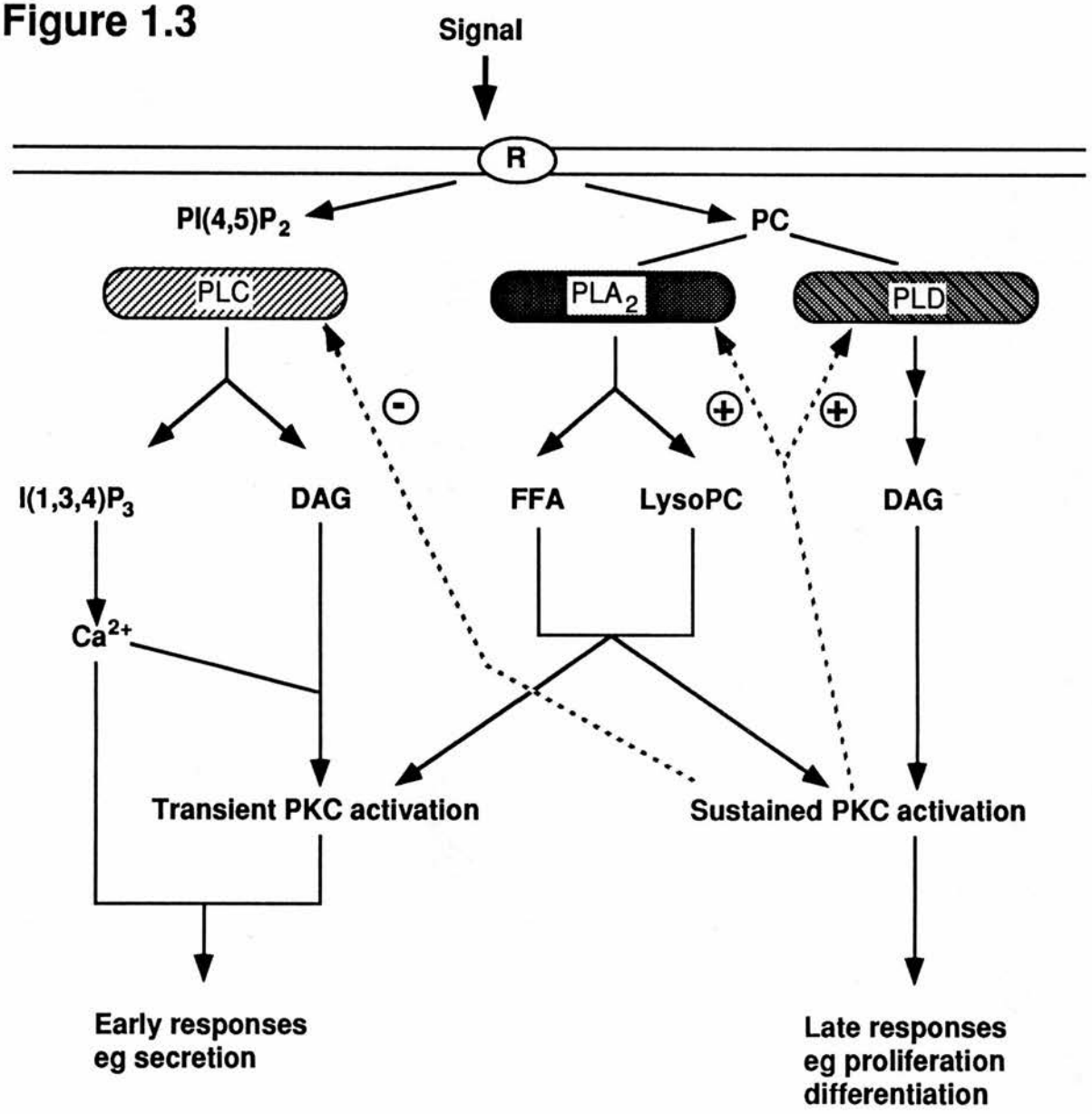

INACTIVE
ACTIVE
LIPIDS
DAG
(Ca2+)
R
P
C
S
PROTEOLYSIS
PROTEOLYSIS
CONSTITUTIVELY
ACTIVE

Figure 1.3 PKC activation following signal-induced membrane phospholipid degradation.

Pathways by which PKC may be activated following stimulus-induced phosphatidyl inositol and phosphatidylcholine hydrolysis. R, receptor; $PI(4,5)P_2$, phosphatidylinositol 4,5-bisphosphate; PC, phosphatidylcholine; I(1,4,5)P3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; FFA, *cis*-unsaturated fatty acid; LysoPC, lysophosphatidylcholine; PKC, protein kinase C. Dashed lines indicate positive and negative feedback of PKC on phospholipase activity. Modified from (Asaoka et al., 1992).

Figure 1.3


Signal
R
PI(4,5)P2
PC
PLC
PLA2
PLD
I(1,3,4)P3
DAG
FFA
LysoPC
DAG
Ca2+
Transient PKC activation
Sustained PKC activation
Early responses
eg secretion
Late responses
eg proliferation
differentiation

Chapter 2

Materials and Methods

2.1 PKC activators and inhibitors

PDBu, mezerein (LC Services Corporation), 1,2 dioctanoyl glycerol (DOG)(Sigma), staurosporine (Calbiochem) and Ro31-8220 (Roche Products Ltd, Welwyn Garden City, UK) were made up as 1-10 mM stock solutions in dimethylformamide (DMF). With the exception of DOG (freshly prepared), all of these reagents were used from stocks maintained at -20°C. H7 was dissolved in distilled water (10 mM stock) and used from aliquots maintained at -20°C.

2.2 Commonly used solutions

5 x agarose gel loading buffer:	0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 1 mM EDTA, 30% glycerol.
10 x TBE:	pH 8.3; 108.9 g/l Tris base, 55.7 g/l boric acid, 4.7 g/l EDTA.
TE:	10 mM Tris/HCl (pH 8), 1 mM EDTA

Tris-saturated phenol: frozen phenol was thawed by bringing to room temperature then heated to 68°C in a water bath. Hydroxyquinoline (0.1%) was added as a preservative and phenol was equilibrated with an equal volume of 0.5 M Tris/HCl (pH 8.0) followed by an equal volume of 0.1 M Tris/HCl (pH 8.0) until buffer pH was > 7.0. Phenol was finally equilibrated with 0.1 volumes of 0.1 M Tris/HCl (pH 8.0) and stored at 4°C under a layer of 0.1 M Tris/HCl (pH 8.0).

2.3 Bacterial culture medium

L-broth:	10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl.
L-agar:	L-broth to which 1% bactoagar was added.
AIX plates:	L-agar was melted over a bunsen burner and left to cool to room temperature. Ampicillin (50 mg/ml), isopropylthio-β-D-galactoside (IPTG)(50 mg/ml) and X-gal (5-bromo-4-chloro-3-indolyl-B-D-galactopyranoside)(4%) were added (1:1000 dilution of each) before plates were poured and allowed to cool at room temperature. Once set, plates were stored for up to 2 weeks at 4°C. Stock solutions of ampicillin, IPTG and X-gal were stored at -20°C.

METHODS

2.4 Cytosolic PKC activity assay

Partially-purified cytosolic PKC activity was determined as the PDBu-induced histone III-S thiophosphorylation in the presence of phosphatidylserine (PS). Partial purification was necessary to remove endogenous inhibitors and phosphatases. A mixed micelle assay was used to enable the Ca^{2+}-dependent and -independent activity to be analysed separately, as PKC activation is obligatorily dependent on phorbol/diglyceride activators under these conditions (Hannun and Bell, 1986). All of the activity measured was PS-dependent as it was absent when PS was replaced by PC, which is unable to act as a cofactor in PKC activation (Nishizuka, 1984b). Thiophosphorylation was measured as ATP-

γ-^{35}S is an acceptable phosphate donor for many protein kinases (Eckstein, 1985) and the products of the reaction are more resistant to subsequent phosphatase activity (Coyne et al., 1987; Wagner and Vu, 1989). However thiophosphorylation occurs at a slower rate than phosphorylation (Wise et al., 1982) and the kinetics of thiophosphorylation, using the method described below, have been shown to be linear up to 30 min (D J MacEwan and R Mitchell; unpublished results).

This method is modified from those in references (Huang et al., 1988; Wise et al., 1982). Tissue from male Wistar rats (230-350 g) was homogenised in 2 volumes of 20 mM Tris/HCl (pH 7.5) containing 50 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF)(Sigma), 0.01% (w/v) leupeptin (Sigma) and 20 µM trans-epoxysuccinyl-l-leucylamido (4-guanidino) butane (E64)(Sigma). Alternatively, COS 7 cells (cultured at 37°C in DMEM with 10% normal calf serum under 5% CO_2) were washed in Ca^{2+}/Mg^{2+}-free Hank's salt solution and then harvested from tissue culture flasks by scraping into ice-cold homogenisation buffer. The suspension was then homogenised using a Ystral polytron homogeniser (Scientific International Industries Ltd, Loughborough, Leics, UK). The homogenate was centrifuged (16,000 ***g***, 20 min, 4°C) and the supernatant was collected and recentrifuged (16,000 ***g***, 5 min, 4°C). The supernatant from the second spin was taken to represent cytosol and was partially purified by loading onto 0.5 ml (cells and pituitaries) or 1.5 ml (other tissues) diethylaminoethyl (DEAE) cellulose (DE52; Whatman International Ltd, Maidstone, Kent, UK) in a Bio-Rad Poly-Prep chromatography column (Bio-Rad Laboratories, Richmond, California, USA) at 4°C. The matrix was then washed with 6 column volumes of homogenisation buffer before the partially-purified PKC was eluted with 3 column volumes of buffer containing 150 mM NaCl. Cytosolic PKC activity

was then measured in an assay mixture (total volume 100 µl) containing, unless otherwise stated, (final concentrations): 10 mM $MgCl_2$, 200 µg/ml PS (sodium salt) (Lipid Products, Nutfield, Surrey, UK), 0.04% Nonidet P-40 (Calbiochem, Novabiochem, Nottingham, UK), 1.25 mg/ml histone III-S (Sigma), 50 µM ATP-γ-[^{35}S](NEN)(0.18 µCi/tube), 1µM PDBu (LC Services Corporation, Scientific Marketing Associates, Barnet, UK) and 25 µl cytosol. Phosphatidylserine vesicles were prepared by drying the lipid from chloroform/methanol under a stream of N_2. The subsequent film of PS was scraped into 20 mM Tris/HCl (pH 7.5) with 0.5 mM EGTA, sonicated, then 0.16% Nonidet P-40 was added. The mixture was vortexed before use. Assay tubes also contained either 600 µM $CaCl_2$ (100 µM free Ca^{2+}) or 5 mM EGTA (less than 3 nM free Ca^{2+}) and inhibitors at various concentrations. All assay components and drugs were dissolved in 20 mM Tris HCl (pH 7.5) with 0.5 mM EGTA except the substrate, histone IIIS, which was dissolved in the $MgCl_2$ solution. Reactions were started by the addition of enzyme, incubated at 30°C for 15 min and stopped by quenching with 20 µl 0.1 M ATP in 0.1 M EDTA (pH 7.0). 50 µl of the quenched reaction mixture was spotted onto a 4 cm^2 piece of P-81 cellulose phosphate ion-exchange chromatography paper (Whatman International Ltd) then washed (3 x 10 ml, 2 min, room temperature) in 75 mM H_3PO_4, dried and counted by liquid scintillation.

2.5 Hydroxyapatite chromatography

PKC isoforms were resolved by hydroxyapatite (HAP) chromatography (Biogel HT, Bio-Rad Laboratories, Watford, Herts, UK), using a procedure based on that published in (Huang et al., 1986a, Kuo et al., 1980) and similar to that described in (Connor and Clegg, 1993). Midbrain or anterior pituitary tissue from male Wistar rats was homogenised

in 6 volumes of ice-cold buffer (25 mM Tris/HCl, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 50 mM 2-mercaptoethanol) containing 1 mM PMSF, 1 mM benzamidine, 2 μg/ml soya bean trypsin inhibitor and 25 μg/ml leupeptin and centrifuged (10 min, 6000 ***g***, 4°C). The resulting supernatant was strained through glass wool and Triton X-100 was added (final concentration 1% (v/v)) before further centrifugation (60 min, 105 000 ***g***, 4°C). The PKCs in the supernatant were partially purified on DEAE cellulose columns as described in Section 2.4 but the elution buffer contained 300 mM NaCl. This fraction was dialysed into 5 mM potassium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM DTT and loaded onto a HAP column equilibrated in the same buffer, which was also used to wash the column (4 volumes). Proteins were eluted in a linear potassium phosphate gradient (5-300 mM; total volume 150 ml, fraction volume 2.5 ml) and the PKC activity was assayed. Fractions were pooled as appropriate, and dialysed against 50% glycerol, 10 mM Tris HCl, 0.5 mM EDTA, 0.5 mM EGTA, 25 mM 2-mercaptoethanol (pH 7.5) for storage at -20 °C.

2.6 Data Analysis

Basal activity with PS alone (at appropriate inhibitor concentrations) was subtracted from the inhibitor curves in the absence/presence of Ca^{2+}. A normalised asymmetric sigmoid Hill curve was fitted to the Ca^{2+} independent evoked activity and subtracted from the evoked activity values obtained in the presence of Ca^{2+}. A normalised curve was then similarly fitted to the resulting values for Ca^{2+}-dependent activity. Curve fitting was carried out using the iterative error-weighted curve fitting program, P.fit (Biosoft, Cambridge, UK).

2.7 Immunoblotting with antibodies to PKC isoforms

Pituitary HAP fractions were diluted 1:1 (v/v) with 50 mM Tris/HCl (pH 7.2), or, for immunostaining of PKC α, β_1, ζ and θ, concentrated 5 fold and resuspended in the same buffer using a Centricon-30 ultrafiltration device (Amicon, Stonehouse, Gloucs, UK) then heated at 100°C for 5 min in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Four µl or, for immunostaining of PKC δ, 1 µl aliquots were applied to 7.5% homogenous microgels and then SDS-PAGE and electroblotting were performed using a PhastSystem apparatus (Pharmacia Biotech, Milton Keynes, Bucks, UK). PKC isoforms were identified with rabbit polyclonal antisera raised to isoform-specific peptide sequences in α, δ, ε and ζ (2 µg/ml)(Gibco BRL, Paisley, Renfrew, UK) and β_1 (2.5 fold dilution)(Marais and Parker, 1989). The specificity of staining with these antisera was confirmed in each case using antibody blocked by preincubation with the relevant antigenic peptide (1 µg/ml). Protein kinase C θ was detected using a mouse monoclonal antibody (1 µg/ml; Transduction Labs, Lexington, KY, USA). Both the midbrain and pituitary HAP fractions were also immunoblotted with a rabbit polyclonal consensus antibody raised to a sequence in the C4 domain conserved between all the PKC isoforms (PKC α:[Ac 543-550-Cys])(Calbiochem). In each case the antibody reaction was visualised with horseradish peroxidase-labelled anti-IgG (Scottish Antibody Production Unit, Carluke, Lanarks, UK) followed by an enhanced chemiluminescence detection system (ECL; Amersham, Aylesbury, Bucks, UK).

2.8 Autophosphorylation

Midbrain and pituitary HAP fractions were incubated at 30°C in a mixture containing 13.3 mM MOPS/KOH, 10.7 mM magnesium acetate, 67 µM [γ-^{32}P] ATP (approximately 10^6 cpm), 6.7 mM DTT, 4.2 mM 2-

mercaptoethanol, 83 μM EDTA, 1 mM EGTA, 1 mM $CaCl_2$, 50 μg/ml PS, 3.3 mM Tris/HCl, 2 μg of BSA and 1 μg/ml protein kinase A peptide inhibitor (Connor and Clegg, 1993). After 30 min, reaction mixtures were heated to 100°C for 5 min in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol and then SDS/PAGE was performed on 15 cm homogenous 8% gels. Phosphoproteins were located by autoradiography after gels had been fixed, stained with Coomassie Blue and destained.

2.9 Preparation of total RNA

RNA was prepared using Nucleon total RNA kit (Version 1.1)(Scotlab, Coatbridge, Strathclyde, UK) according to the manufacturer's instructions. Fresh or frozen tissue was homogenised in lysis solution (2 ml/100 mg tissue), using a polytron homogeniser. 600 μl of deproteinisation solution was added per 1 ml of lysis solution, tubes were vortexed and incubated on ice for 5 min. 24:1 chloroform:isoamyl alcohol (1.4 ml/ml of lysis solution) was added and after vortexing, tubes were incubated on ice for a further 10 min. After centrifugation (10 000 ***g***, 5 min, 4°C), the upper aqueous phase was transferred to a fresh RNAse-free tube and the chloroform extraction procedure was repeated, nucleon silica suspension (700 μl/ 15 ml tube) being added following centrifugation. Further centrifugation (1400 ***g***, 3 min, 4°C) was then required to compact the silica at the interface before the aqueous phase was again transferred to a fresh tube. One volume of 100% ethanol (-20°C) was added and after incubation for 30 min at -20°C, nucleic acids were pelleted (12 000 ***g***, 10 min, 4°C) and washed with 70% ethanol, centrifuged (12 000 ***g***, 10 min, 4°C) and dried at room temperature for 10 min. After resuspending in 180 μl RNAse free water, 20 μl DNase restriction buffer and 2 μl DNAse was added. Tubes were incubated at 37°C for 15 min before the RNA was precipitated using 0.5 volumes ammonium acetate

(7.5 M) and 2.5 volumes 100% ethanol (-20°C). The RNA was then stored at -70°C until required. Immediately prior to use, RNA was pelleted (10 000 ***g***, 10 min, 4°C), washed with -20°C 70% ethanol (500 µl), repelleted (10 000 ***g***, 5 min, 4°C) and dried at room temperature for 10 min. It was then resuspended in the appropriate volume of RNAse free water.

2.10 PCR techniques

2.10.1 Reverse transcriptase PCR

Fragments were amplified from total RNA by reverse transcriptase PCR (RT-PCR), using the Perkin-Elmer Cetus *GeneAmp* RNA PCR kit according to the manufacturer's instructions. In all cases, random hexamers were used to reverse transcribe all the RNA. A master mix was prepared containing (final concentrations): 5 mM $MgCl_2$, 1 x PCR buffer II (Mg^{2+} free buffer), dNTPs (each 1 mM), 1 U/µl RNase inhibitor, 2.5 µM random hexamers, and sterile distilled water (dH_2O) to a final volume of 18 µl per sample. This was then added to 2 µl total RNA (containing 1µg) and the mix was overlaid with mineral oil to prevent evaporation. Tubes were incubated at room temperature for 10 min to allow the extension of the hexameric primers by reverse transcriptase, before being heated to 42°C for 15 min, 99°C for 5 min and then cooled 5°C for 5 min. A PCR master mix containing (final concentrations): 2 mM $MgCl_2$, 1 x PCR II buffer, 2.5 U/100 µl *AmpliTaq* DNA polymerase and sterile dH_2O to a final volume of 78 µl was added to each tube by pipetting under the oil. One µl of each primer (final concentration 0.15 µM) was then added to give a total volume of 100 µl. The PCR cycling was conducted according to conditions determined for each pair of primers. In general, the melting temperature (Tm) for each oligonucleotide primer was calculated using the equation:

$$Tm = 81.5 + 16.6 (\log [Na^+]) + 0.41 (\text{fraction G +C}) - (600/N)$$

N=length of oligonucleotide

Initial PCR amplification was usually conducted annealing at a temperature approximately 5°C below the Tm for the two primers. The annealing temperature for subsequent reactions was altered according to the products initially contained. To increase the stringency of the PCR, the annealing temperature was raised by 3-10°C.

2.10.2 PCR from cDNA libraries

5-10 µl cDNA library (10^{10} bacteriophage/ml) was heated to 95°C for 10 min then cooled to 4°C over a period of 15 min, to denature the DNA. DNA was amplified in a mix containing (final concentrations): denatured cDNA library, sense and antisense primers (each 140-350 nM), 1 x PCR buffer, 100 µM dNTPs and double dH_20 to a final volume of 99.5 µl. The mix was overlaid with mineral oil (Sigma) and the PCR cycling was commenced, according to conditions determined for each pair of primers, as described in Section 2.10.1. When the temperature reached 94°C at the beginning of the first cycle, 2.5 U Taq polymerase was pipetted under the mineral oil. This 'hot start' procedure, adding Taq polymerase only once the temperature reached 94°C, was used to ensure that polymerisation could not occur before the primers had annealed to their specific target sequence. After amplification was complete, 5-20 µl of the PCR mix was analysed by agarose gel electrophoresis (Section 2.18.2) to determine the products.

2.11 Purification of DNA fragments

2.11.1 Wizard purification

The Wizard DNA clean-up system (Promega) was used for the separation of DNA fragments from small nucleotides, proteins and salts, according to the manufacturer's instructions. Samples (volume 50-500 µl)

were added to 1 ml of Wizard DNA clean-up resin and mixed by inverting. The resin containing the bound DNA was pipetted in the barrel of a 3 ml disposable syringe with a Wizard minicolumn attached. The slurry was pushed into the minicolumn using the plunger then the column was washed by pushing through 2 ml isopropanol (80%). The syringe was removed and the minicolumn centrifuged (20 sec, 12 000 ***g***) to dry the resin. After leaving at room temperature for 5-15 min to allow the isopropanol to evaporate, the DNA was eluted by applying 50 µl prewarmed water or TE (65-70°C), leaving for 1 min, then centrifuging (12 000 ***g***). The purified DNA was stored at -20°C.

2.11.2 Gel Purification

To separate the required PCR product from other DNA fragments, the Geneclean II kit was used according to the manufacturer's instructions. Fragments were excised from a 1% TBE agarose gel and the gel slice weighed to determine the volume. 4.5 volumes of sodium iodide and 0.5 volumes of TBE modifier (as provided in the kit) were added and the agarose was melted by incubation at 45-55°C for 5 min. 5-10 µl glassmilk (silica matrix suspension) was added, depending on the mass of DNA present, and the solution was incubated on ice for 5 min to allow the DNA to bind to the silica. The glassmilk/DNA complex was pelleted by centrifugation for approximately 5 sec and the supernatant discarded before the pellet was washed 3 times by resuspending in approximately 10 volumes of ice cold NEW wash solution (NaCl/ethanol/water, in Tris/EDTA pH7-8.5, as provided in the kit). The tube was then respun and the remaining supernatant removed to ensure that the eluate was not diluted with residue wash solution. DNA was eluted by resuspending the pellet in an equal volume of TE or water and incubation at 45-55°C for 2-3 min

before centrifuging for 30 seconds to pellet the resin. The supernatant containing the eluted DNA was removed and retained, and the elution procedure repeated to maximise the recovery.

2.12 Ethanol precipitation

Before the purified DNA fragments could be ligated into a cloning vector, ethanol precipitation was necessary to concentrate the DNA. 0.1 volumes 3 M sodium acetate were added, samples were vortexed and 2.5 volumes ethanol were added. Samples were then incubated in a dry ice/ethanol bath for 15-20 min or overnight at -20°C. DNA was pelleted by spinning for 10 min in an Eppendorf bench top centrifuge at room temperature and washed to remove the salts in 500 µl 70 % ethanol. Pellets were dried at 37°C for 10 min and resuspended in an appropriate volume of TE or water.

2.13 Ligation of DNA fragments into plasmid vector

Ligation reactions were performed in a total volume of 10 µl, containing 1 µl 10 x ligation buffer (0.5 M Tris/HCl pH 7.6, 100 mM $MgCl_2$, 10 mM DTT), 1 mM ATP, 1 U T4 DNA ligase (BCL) and a 1:1-1:5 molar ratio of vector DNA:insert DNA. Typically, approximately 50-100 ng of vector DNA was used. Ligation reactions were incubated at 10-25°C overnight before being transformed into *E.coli* as described below.

2.14 Preparation and transformation of competent *E. coli*

A single colony of Escherichia coli of strains DH5α or JM109 was grown overnight in 3 ml L-broth, diluted into 40 ml L-broth and grown in an orbital shaker at 37°C until OD_{540} reached 0.3. Cells were pelleted by centrifugation (7000 rpm, 5 min, 4°C), placed on ice, and resuspended in 0.25 volumes ice cold 0.1 M $MgCl_2$. Cells were pelleted as before,

resuspended in 0.05 volumes $MgCl_2$ and left on ice for 45 min. After pelleting as before, cells were resuspended in the same volume of sterile storage buffer (0.1 M MOPS (pH 6.5), 50 mM $CaCl_2$, 20% glycerol) and left on ice for a further 20 min to acquire full competence. Competent cells were aliquoted into 1.5 ml eppendorf tubes, snap frozen in a dry ice bath and stored at -70°C.

To transform cells, ligation products were diluted to a volume of 60 µl, added to 100-200 µl competent cells and incubated on ice for 30 min. Cells were then heated shocked at 42°C for 2 min in a water bath and incubated on ice for 10 min. Cells were added to 2 ml L-broth and incubated at 37°C for 2 hours. Cells were pelleted, resuspended in 100 µl L-broth and spread on AIX plates, prepared as described in Section 2.3. Plates were incubated overnight at 37°C. These plates contain X-gal, which may be degraded by β galactosidase. Colonies which contain the DNA fragment are unable to produce β galactosidase so remain white, while colonies containing plasmid vector but no DNA fragment turn blue, due to degradation of the X-gal. White colonies were picked for mini-prep analysis as described below.

2.15 Small scale preparation of plasmid DNA

Plasmid DNA was prepared by the alkaline lysis method. Single, white colonies of *E coli* were picked from AIX plates and grown overnight in 3 ml L-broth containing ampicillin (50 µg/ml) at 37°C in a rotator. 1.5 ml of this culture was used to prepare plasmid DNA. Cells were pelleted by centrifuging for 1 min and resuspended in 100 µl GTE solution (50 mM glucose, 25 mM Tris, 10 mM EDTA). After 5 min at room temperature, 200 µl of alkaline SDS solution (1% SDS, 0.2 N sodium hydroxide) was added. Tubes were mixed, incubated on ice for 5 min, and 150 µl ice-cold 3 M potassium acetate containing 11.5% (v/v) glacial acetic acid was added,

followed by a further incubation on ice. Tubes were then spun in a bench top centrifuge for 7 min and the supernatant was transferred to a fresh tube. A phenol/chloroform extraction was then performed using 0.5 volumes Tris-saturated phenol (prepared as described in Section 2.2) and 0.5 volumes chloroform:isoamyl alcohol (24:1, v/v). Plasmid DNA was precipitated from the upper aqueous with 0.7 volumes of isopropanol and washed in 70% ethanol. After air drying (10 min, 37°C), the pellet was resuspended in either 40 µl TE containing RNase (20 µg/ml) for subsequent restriction analysis, or 20 µl sterile dH_2O, for sequencing. Plasmid DNA was stored at -20°C.

2.16 Restriction enzyme digestion of DNA

DNA was digested using 1 unit of restriction enzyme per 1 µg of DNA. Reaction buffer, volume as appropriate to give the dilution specified by the manufacturer, and dH_2O were added to a final volume large enough to give a final enzyme dilution of 1:10. This reduced the glycerol, in which the enzyme is stored, to a level at which it would not inhibit digestion. Reactions were incubated at 37°C, unless otherwise specified by the enzyme supplier, for 2-4 hours before the resulting DNA fragments were separated by agarose gel electrophoresis (Section 2.18.2).

2.17 DNA sequencing

Plasmid DNA was sequenced by the dideoxy method, using the Sequenase version 2.0 kit (USB) according to the manufacturer's instructions. DNA samples (2-5 ng) were made up to a volume of 60 µl with sterile dH_2O and denatured by the following method. 15 µl 2 M sodium hydroxide was added and tubes were incubated for 10 min at 37°C. After addition of 15 µl sodium acetate (3 M) and vortexing, DNA was precipitated in 300 µl absolute ethanol, either in a dry ice/ethanol bath for 15 min or

overnight at -20°C. The precipitate was then collected by centrifugation for 10 min and washed in 70% ethanol before being raised in 7 µl dH_2O. The appropriate primer was annealed to the denatured DNA in a final volume of 10 µl containing 2 µl sequenase reaction buffer (200 mM Tris HCl pH 7.5, 100 mM $MgCl_2$, 250 mM NaCl) and 10 ng primer by heating to 85°C followed by cooling to room temperature. Plasmid DNA was sequenced using the SP_6 promoter, T7 or M13 forward primers provided with the Sequenase version 2.0 kit. The products of asymmetric PCR were sequenced using the primer which was limiting in the PCR reaction. Annealed DNA samples were then extended and labelled in an incubation mix containing 1 µl 0.1 M DTT, 2 µl labelling mix (diluted 1:5 in dH_2O from a solution containing dCTP, dGTP and dTTP (7.5 µM each)), 5 µCi ^{35}S dATPαS (Amersham, 1000 Ci/mMol) and 2 µl Sequenase Version 2.0 (diluted 1:8 in a buffer containing 10 mM Tris/HCl (pH 7.5), 5 mM DTT, 0.5 mg/ml BSA) at room temperature for 2-5 min. 3.5 µl aliquots were then added to 4 tubes each containing 2.5 µl of termination mix for one nucleotide (containing 8 µM ddNTP) and incubating at 37°C for 5 min before 4 µl stop solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol FF) was added. Products were then separated by polyacrylamide/urea gel electrophoresis.

2.18 Electrophoretic techniques

2.18.1 Denaturing polyacrylamide/urea gel electrophoresis

6% denaturing polyacrylamide gels were prepared by dissolving 42 g urea (Aristar, BDH), 20 ml of 30% (33:1) acrylamide:bisacrylamide (BDH, premixed) and 10 ml TBE pH 8.8 (10x) in a final volume of 100 ml. The solution was filtered through Whatman membrane filters (0.45 µM pore size) under vacuum and degassed. 70 µl TEMED (N,N,N',N',-teramethylethylene

diamine) and 70 µl ammonium persulphate (APS)(25%) was added to 70 ml gel mix immediately before the solution was poured between 300 mm x 400 mm x 0.4 mm glass plates previously sealed with 5% agarose. 20-tooth combs (0.4 mm) were inserted and the gels left to polymerise. Gels were then attached to vertical gel electrophoresis apparatus (Gibco BRL, Model S2) containing 0.5 x TBE buffer and pre-electrophoresed at 1200 V for 30 min prior to loading. Samples were boiled for 2 min, 4 µl were loaded into the wells and the gel electrophoresed at 1500 V for 3-4 hours. Gels were fixed for 30 min (5% methanol, 15% acetic acid) before being transferred to Whatman No. 1 paper, covered with Saran wrap and dried using a heated vacuum gel drier (Biorad Model 583). The dried gel was then exposed to autoradiographic film (Fuji RX X-ray film), typically for 16 hours at room temperature. Films were developed for 2 min in Kodak LX-24 developer (4 fold dilution), fixed for 2 min in Kodak FX-40 fixer (4 fold dilution), rinsed in water and dried.

2.18.2 Horizontal agarose gel electrophoresis

1% (w/v) agarose (Agarose MP, Boehringer) gels containing 0.5 mg/ml ethidium bromide were made up in 1 x TBE. 100 ml horizontal gel slabs (140 mm x 110 mm x 30 mm) or 25 ml mini agarose gels (83 mm x 57 mm x 30 mm) were placed in gel electrophoresis apparatus (Gibco BRL, Model HS or Gibco BRL, Model Horizon 58 respectively) containing sufficient 1 x TBE buffer to cover the gel. Samples were loaded in 0.2 volumes of loading buffer and gels were usually electrophoresed at 100 V for as long as required to see sufficient band separation. DNA fragments were visualised using a long wave UV transilluminator. DNA was photographed using Polaroid film type 667 in a Polaroid Cu-5 camera.

Figure 2.1 Flow diagram of the basic procedure used to measure partially purified PKC activity.

Unless otherwise stated, PKC was partially purified from tissues by this method. In cases where peptide substrates replaced histone IIIS, reactions were stopped by the addition of TCA and the precipitated pelleted by centrifugation (16 000 ***g***, 5 min, 4°C) before spotting a sample of the supernatant onto P81 paper.

Figure 2.1

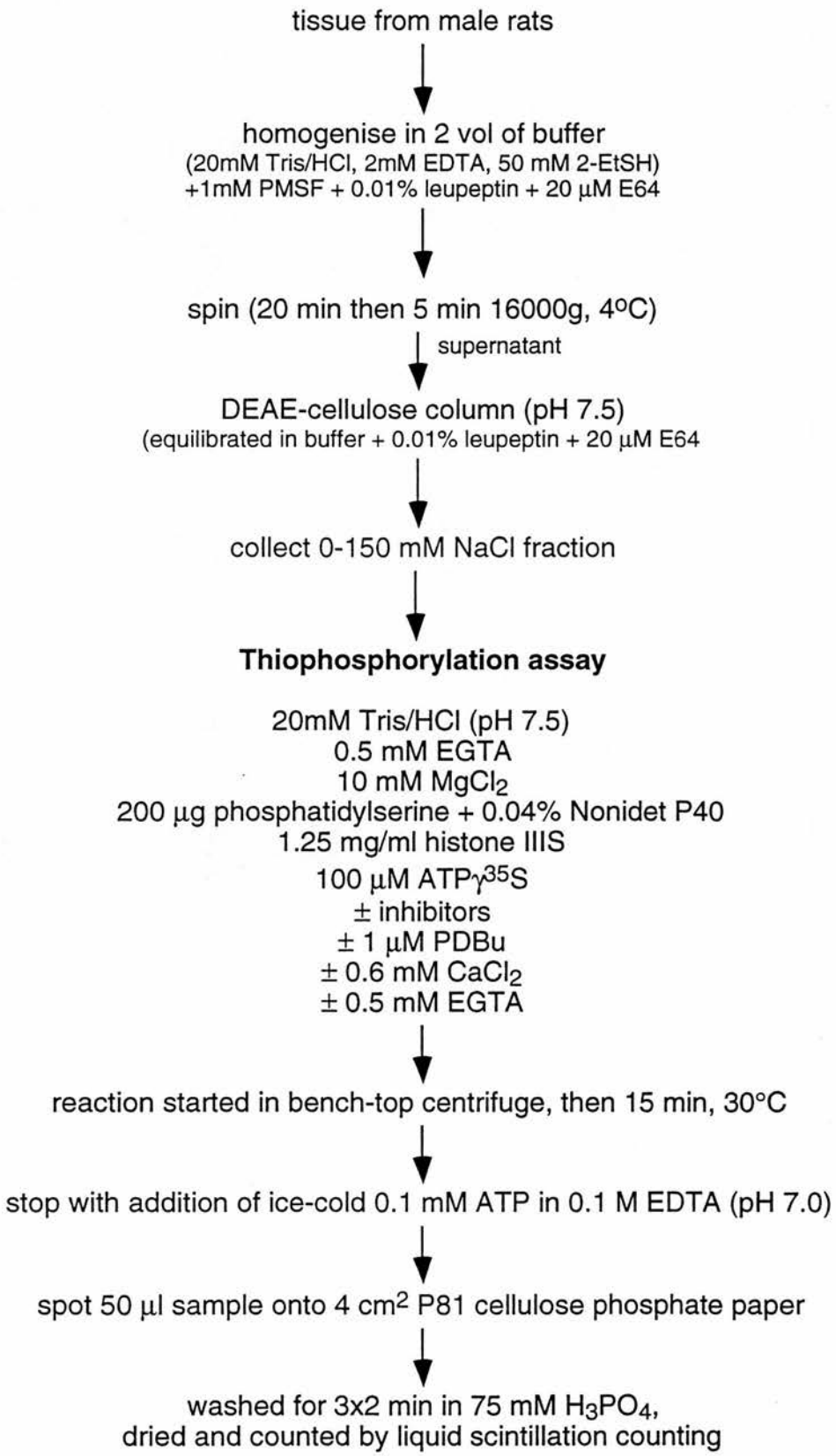
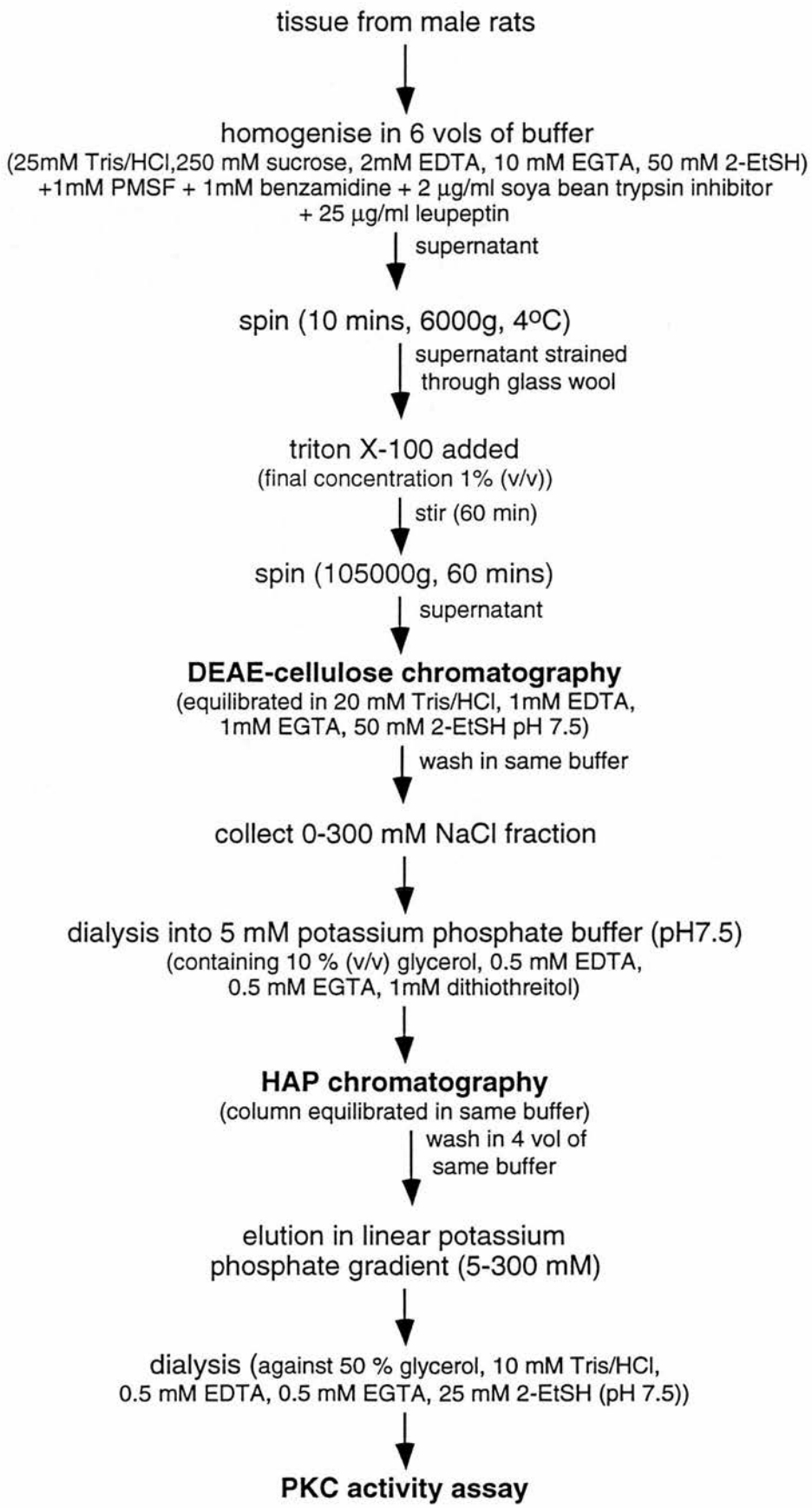

tissue from male rats
homogenise in 2 vol of buffer
(20mM Tris/HCl, 2mM EDTA, 50 mM 2-EtSH)
+1mM PMSF + 0.01% leupeptin + 20 μM E64
spin (20 min then 5 min 16000g, 4ºC)
supernatant
DEAE-cellulose column (pH 7.5)
(equilibrated in buffer + 0.01% leupeptin + 20 μM E64
collect 0-150 mM NaCl fraction
Thiophosphorylation assay
20mM Tris/HCl (pH 7.5)
0.5 mM EGTA
10 mM MgCl2
200 μg phosphatidylserine + 0.04% Nonidet P40
1.25 mg/ml histone IIIS
100 μM ATPγ35S
± inhibitors
± 1 μM PDBu
± 0.6 mM CaCl2
± 0.5 mM EGTA
reaction started in bench-top centrifuge, then 15 min, 30°C
stop with addition of ice-cold 0.1 mM ATP in 0.1 M EDTA (pH 7.0)
spot 50 μl sample onto 4 cm2 P81 cellulose phosphate paper
washed for 3x2 min in 75 mM H3PO4,
dried and counted by liquid scintillation counting

Figure 2.2 **Flow diagram of the procedure used for purification of PKC by HAP chromatography.**

Figure 2.2


tissue from male rats
homogenise in 6 vols of buffer
(25mM Tris/HCl,250 mM sucrose, 2mM EDTA, 10 mM EGTA, 50 mM 2-EtSH)
+1mM PMSF + 1mM benzamidine + 2 µg/ml soya bean trypsin inhibitor
+ 25 µg/ml leupeptin
supernatant
spin (10 mins, 6000g, 4ºC)
supernatant strained through glass wool
triton X-100 added
(final concentration 1% (v/v))
stir (60 min)
spin (105000g, 60 mins)
supernatant
DEAE-cellulose chromatography
(equilibrated in 20 mM Tris/HCl, 1mM EDTA,
1mM EGTA, 50 mM 2-EtSH pH 7.5)
wash in same buffer
collect 0-300 mM NaCl fraction
dialysis into 5 mM potassium phosphate buffer (pH7.5)
(containing 10 % (v/v) glycerol, 0.5 mM EDTA,
0.5 mM EGTA, 1mM dithiothreitol)
HAP chromatography
(column equilibrated in same buffer)
wash in 4 vol of same buffer
elution in linear potassium phosphate gradient (5-300 mM)
dialysis (against 50 % glycerol, 10 mM Tris/HCl,
0.5 mM EDTA, 0.5 mM EGTA, 25 mM 2-EtSH (pH 7.5))
PKC activity assay

CHAPTER 3

CHARACTERISATION OF NATIVE PKC ACTIVITY FROM DIFFERENT TISSUE SOURCES

3.1 INTRODUCTION

There are currently known to be eleven different isoforms of PKC (Nishizuka, 1992), which have been found to exhibit unique tissue distributions (Wetsel et al., 1992), as well as varying in their biochemical properties. The cPKCs α, β and γ are classified as being dependent on Ca^{2+} for activation (Nishizuka, 1992), although there is evidence to suggest that these isoforms may be stimulated by some PKC activators, for example TPA, in the absence of Ca^{2+}(Ryves et al., 1991). Furthermore the presence of unsaturated fatty acids has been shown to lower the Ca^{2+} dependence of the cPKCs (Shinomura et al., 1991), and it has been reported that autophosphorylation may produce Ca^{2+}-independent forms of PKC β (Pelech et al., 1991).

Isoforms have also been found to vary in their phospholipid dependence (Bazzi and Nelsestuen, 1987) and activation by diglycerides and phorbol esters (Kazanietz et al., 1993; Ryves et al., 1991). Although all isoforms are activated by PS, individual isoforms are potently stimulated by other phospholipids. PKC δ, for example, was strongly activated by phosphatidyl inositol (PI) in the presence of phorbol esters (Mizuno et al., 1991), while there is evidence that PKC ε shows a preference for cardiolipin (Konno et al., 1989). It has been suggested that PKC η may be strongly activated by cholesterol sulphate in the absence of phorbol esters (Ikuta et al., 1994), while PKC α and βI may be activated by PIP_2 in place of phorbol esters, and PI can act as a cofactor for these isoforms in place of PS (Kochs et al., 1993a). Furthermore cis-unsaturated fatty acids, such as arachidonic acid, act synergistically with diglycerides and PS in the activation of the cPKCs while arachidonic acid has an inhibitory effect on PKC δ. (Ogita, 1992). Most evidence suggests that the aPKCs ζ, ι and μ are not activated

by diglycerides and phorbol esters (McGlynn et al., 1992; Nakanishi and Exton, 1992; Pfizenmaier et al., 1993; Selbie et al., 1993) and expression of PKC ζ in baculovirus showed that this isoform is unable to bind PDBu and the highly potent PKC ligand bryostatin even at high concentrations (Kazanietz et al., 1993). These isoforms are instead constitutively active in the presence of phospholipids (Liyanage et al., 1992; McGlynn et al., 1992).

There are also reports that the PKC isoforms vary extensively in their specificity for different substrates, both synthetic and endogenous, as detected in *in vitro* kinase activity assays. The nPKCs and cPKCs are thought to be more restricted than the cPKCs in the substrates that they can efficiently phosphorylate, and there are reports that these isoforms show little activity on histone (Schaap and Parker, 1990; Schaap et al., 1989), a commonly used substrate for *in vitro* activity assays.

In view of the variations in the properties of the PKC isoforms, and the differences detected in different assay systems, it was decided to compare the properties of PKC partially-purified from a variety of tissue sources with those of the H7-resistant PKC from anterior pituitary, investigated under the same conditions. This form of PKC has been detected previously in studies of stimulus-secretion coupling in the anterior pituitary (Johnson et al., 1992b, Johnson et al., 1993; Thomson et al., 1993b) as well as *in vitro* in cytosol partially purified from this tissue. A mixed micelle assay system was selected as this enables the Ca^{2+}-dependent and -independent activity to be analysed separately and PKC activation is obligatorily dependent on phorbol/diglyceride activators under these conditions (Hannun and Bell, 1986). A variety of tissue and cell lines, chosen for their isoform content, were used as sources of PKC. Midbrain was investigated because it is reported to contain all of the well-characterised PKC isoforms (Scott-Young, 1989), while the other tissues were studied because of their reported

enrichment in particular isoforms. The COS 7 fibroblast cell line was used as these cells are reported to contain only PKC α and ζ (Kosaka et al., 1988; Ways et al., 1992).

Protein kinase C activity was evoked by either of the diterpenes, PDBu and mezerein, or by the synthetic diglyceride, 1,2-dioctanoyl-sn-glycerol (DOG), as these activators have been shown to differ in their ability to activate various PKC-mediated processed *in vivo* (Johnson et al., 1992; MacEwan and Mitchell, 1991; MacEwan et al., 1991). For example, PDBu elicits inverse effects on depolarisation-induced Ca^{2+} influx through L-type Ca^{2+} channels, causing facilitation in anterior pituitary pieces while having an inhibitory effect in the GH_3 clonal pituitary cell line (MacEwan and Mitchell, 1991). Dioctanoyl-*sn*-glycerol, however, was only able to elicit the facilitatory response, whereas mezerein caused a facilitatory response in both tissues. As, in each case, the facilitatory response was relatively resistant to H7, it was suggested that mezerein may be a selective activator of the H7-resistant form of PKC (Mitchell et al., 1992). There is also evidence to support this from studies of LH release from anterior pituitary tissue. Both PDBu and mezerein induce LH release that is inhibited by H7 in a biphasic manner, but the H7-resistant component represents a larger proportion of the release induced by mezerein than by PDBu (Thomson et al., 1993b).

Thus the biochemical properties of the H7-resistant kinase were characterised and compared with those of PKCs from a variety of tissues of known isoform content to determine whether the H7-resistant kinase could be identified as any of the eleven PKC isoforms already characterised.

3.2 Specific Methodology

Tissue from male Wistar rats was homogenised, centrifuged and partially purified by DEAE cellulose chromatography, as described in Section 2.4. Tissue from one organ was used, except in the case of anterior pituitary tissue, when 10 rat or 3 sheep pituitaries were taken. Alternatively, COS 7 cells (cultured at 37°C in DMEM with 10% normal calf serum under 5% CO_2) were washed in Ca^{2+}/Mg^{2+}-free Hank's salt solution and then harvested from tissue culture flasks by scraping into ice-cold buffer before homogenization. Protein kinase C activity was measured as described in Section 2.4. Unless otherwise stated the substrate for thiophosphorylation was histone IIIS. The assay procedure was identical when myelin basic protein (MBP) was used as the substrate. When peptide substrates were used, the method in Section 2.4 was modified slightly. The reaction was terminated by addition of 20 µl trichloroacetic acid (TCA)(6.8% w/v final). After incubation on ice for 15 min, TCA-precipitable material was removed by centrifugation and the supernatant was spotted onto 2x2 cm pieces of P-81 cellulose phosphate ion-exchange chromatography paper (Whatman International Ltd, Maidstone, Kent, UK), which were washed extensively in 75 mM H_3PO_4, dried and counted by liquid scintillation. The sequences of the peptide substrates used are as follows:

PKC α pseudosubstrate peptide (RFARKGSLRQKNV)

PKC ε pseudosubstrate peptide (ERMRPRKRQGSVRRRV)

PKC ζ pseudosubstrate peptide (EDKSIYRRGSRRWRKL)

glycogen synthase-(1-12) peptide (PLSRTLSVAAKK)

$AcMBP_{(4-14)}$ peptide (AcQKRPSQRSKYL)

histone H1 phosphorylation site peptide (RRKASGP)

3.3 RESULTS

Activation of PKC by phorbol 12, 13-dibutyrate

Protein kinase C activity in anterior pituitary cytosol, measured as histone IIIS thiophosphorylation, was evoked by PDBu in a concentration-dependent manner (10 nM-3 μM), in both the absence (<3 nM) and presence (100 μM free) of Ca^{2+}, as shown in Figure 3.1. This reaction was specific as no activation was detected when 4 β-PDBu was replaced by the essentially inactive stereoisomer, 4 α-PDBu (Figure 3.1). The EC_{50} values (concentration at which the activator achieved 50% of the maximal response) were 637 ± 311 nM and 380 ± 117 nM in the absence and presence of Ca^{2+} respectively. This was compared with the EC_{50} values for a number of other tissues as shown in Table 3.1. While the potency of PDBu varied between tissues, particularly in the absence of Ca^{2+}(EC_{50} values in the range 620-2270 nM), in all cases PDBu was more potent in the presence of Ca^{2+} (EC_{50} values 77-380 nM). There were also variations in the proportion of the maximum activity occuring in the absence of Ca^{2+}, with only 48% in COS 7 cells, in which PKC α is the only phorbol ester responsive isoform (Kosaka et al., 1988), while 80% of the activity in spleen, which contains predominantly PKC α, β and δ (Johnson et al., 1994), was Ca^{2+}-independent (Table 3.1). For subsequent inhibition studies, PKC activity was elicited by 1μM PDBu as at this concentration, in most tissues, the response was almost maximal for both Ca^{2+}-dependent and Ca^{2+}-independent activity, with typical values in midbrain of 5.6 ± 0.4 and 12.0 ± 0.3 x 10^3 dpm per mg tissue equivalent. The corresponding values for anterior pituitary were 3.0 ± 1.3 and 3.6 ± 1.9 x 10^3 dpm per mg tissue equivalent.

Effects of different PKC inhibitors

The effects of different PKC inhibitors on PDBu-evoked PKC activity from rat midbrain, anterior pituitary and COS 7 cells was studied. The Ca^{2+}-dependent and Ca^{2+}-independent activity was compared in both tissues, while in COS 7 cells the PKC activity evoked by 1 μM PDBu was entirely Ca^{2+}-dependent. Table 3.2 shows IC_{50} values (the concentration required to inhibit 50% of the effect) for inhibition by H7, staurosporine and Ro 31-8220. In midbrain, staurosporine, Ro 31-8220 and H7 all inhibited both Ca^{2+}-dependent and -independent activity with similar IC_{50} values (0.1-0.1.5 μM, 0.18-0.19 μM and 20-30 μM respectively, as shown in Table 3.2). The potencies of all three inhibitors on activity from COS 7 cells were similar to those obtained in midbrain. This is in contrast to anterior pituitary where, although staurosporine and Ro31-8220 inhibited Ca^{2+}-dependent and -independent activity with similar potencies, H7 was considerably less potent on Ca^{2+}-independent activity (IC_{50} values of 148 ± 38 and 25 ± 4 μM for Ca^{2+}-independent and -dependent respectively).

Regional differences in H7 inhibition of PKC

In order to determine the tissue distribution of the H7-resistant PKC activity, the effect of H7 on PDBu-evoked PKC activity from a variety of tissues and cell lines was studied and the IC_{50} values are shown in Table 3.3. In the majority of the regions investigated, there was no difference in the potency of H7 on the Ca^{2+}-dependent and -independent activity evoked by PDBu, the IC_{50} values ranging from 10 to 45 μM. However, in cytosol from anterior pituitary and perhaps to a lesser extent lung, the Ca^{2+}-independent but not -dependent activity was relatively insensitive to H7 (IC_{50} values for Ca^{2+}-independent activity of 145 ± 38 μM and 87 ± 37 μM respectively, while those for Ca^{2+}-dependent activity were 25 ± 4 μM and 39 ± 6 μM

respectively). Unless otherwise stated, the tissues studied were obtained from male Wistar rats. However sheep anterior pituitaries were also investigated, to determine whether the H7 resistant PKC activity was present in other mammalian species and to evaluate their potential as a source of this form of PKC for further studies. As shown in Table 3.3, PDBu-evoked Ca^{2+}-independent activity in sheep anterior pituitaries was indeed relatively resistant to H7 (IC_{50} value 128 ± 13 μM) although no Ca^{2+}-dependent activity was detected in this tissue.

In view of the possibility that mezerein may represent a selective activator of the H7 resistant PKC (Mitchell et al., 1992; Thomson et al., 1993b), and therefore allow detection of H7-resistant activity in tissues containing only small quantities of this kinase, the inhibition by H7 of mezerein-evoked activity from a number of tissues was investigated. However, as shown in Table 3.4, Ca^{2+}-independent activity in midbrain, thalamus and spleen was sensitive to H7 (IC_{50} values in the range 25-40 μM); the potency of H7 on mezerein-evoked activity being similar to that for PDBu-evoked activity in these tissues. H7-resistant activity in pituitary and lung was activated by mezerein (IC_{50} values 148 ± 21 and 75 ± 10 respectively), the potency of H7 being similar for both mezerein and PDBu-evoked activity.

Protein kinase C activity evoked by mezerein and PDBu did, however, vary in Ca^{2+}-dependency. In anterior pituitary, maximal activity was evoked by mezerein (1 μM) in the absence of Ca^{2+} and thus no separate determination of the potency of H7 on Ca^{2+}-dependent mezerein-evoked activity could be made. The maximal amount of PKC activity was similar with both PDBu and mezerein, the only difference being the Ca^{2+} dependence. This is consistent with previous reports that with some activators, cPKCs do not display rigorous Ca^{2+} dependence (Ryves et al., 1991), and we have

evidence to suggest that mezerein is more potent than PDBu at activating at least PKC α in the absence of Ca^{2+}(Johnson et al., 1994).

As there is evidence to suggest that histone IIIS is a poor substrate for the Ca^{2+}-independent PKC isoforms (Schaap and Parker, 1990; Schaap et al., 1989), the effect of H7 on PDBu-evoked activity was investigated using an alternative protein substrate, myelin basic protein (MBP). This protein is a better substrate for most of the Ca^{2+}-independent PKCs and is a particularly good substrate for PKC δ (Kazanietz et al., 1993). The resulting IC_{50} values are shown in Table 3.5. In most tissues the potency of H7 on PDBu-evoked MBP thiophosphorylation was similar to that obtained for histone IIIS thiophosphorylation, although the total amount of activity detected was much higher. For example, in midbrain, detectable PDBu-evoked PKC activity was approximately 3 fold greater for MBP than histone IIIS, with typical values of 53.5 and 17.6 x 10^3 dpm per mg tissue equivalent respectively. However in anterior pituitary cytosol, Ca^{2+}-independent activity was sensitive to H7 (IC_{50} value 34 ± 7 μM), suggesting that this substrate is more selective for the other Ca^{2+}-independent PKC isoforms in pituitary than for the H7-resistant kinase. H7-resistant MBP phosphorylation was detectable, however, in cytosol from skin taken from the ears (IC_{50} value 150 ± 30 μM), although all the PDBu-evoked activity in this tissue was Ca^{2+}-independent. The IC_{50} value for this tissue could not be determined using histone IIIS as the substrate as there was no activity detectable.

Effect of substrate on PKC activity from rat midbrain

In view of the differences previously seen in the properties of PKC activity from some tissues with different substrates, and reports that histone is a poor substrate for some PKC isoforms (Schaap and Parker, 1990; Schaap et al., 1989), the H7 inhibition of midbrain PKC activity was compared using

a variety of protein and synthetic peptide substrates. Figure 3.2 shows the relative kinase activity detected and the Ca^{2+}-dependence with each substrate. In some case accurate K_m values for some of these substrates cannot be consistently obtained (Kazanietz et al., 1993) so a fixed high concentration of substrate was used, as has been done in previous studies (Kazanietz et al., 1993; Koide et al., 1992). The total amount of midbrain PKC activity detected varied greatly with the phosphate acceptor, the highest activity occurring with the glycogen synthase (GS) peptide, which was six times greater than the histone IIIS thiophosphorylation activity. The peptides based on PKC phosphorylation sites from histone and MBP (Yasuda et al., 1990) were the poorest substrates tested, with the histone peptide showing little detectable phosphorylation. This is consistent with a recent report that all the cPKCs and PKC δ, ε, η and ζ expressed in baculovirus show no detectable activity with this substrate (Kazanietz et al., 1993). The relative proportion of the midbrain PKC activity that was dependent on Ca^{2+} differed considerably when using different substrates. In general the peptide substrates showed the least Ca^{2+} dependence, with the majority of the activity occuring in the absence of Ca^{2+}. However with the $AcMBP_{(4-14)}$ peptide, the converse was true with only one fifth of the total activity occurring in the absence of Ca^{2+}, although with the MBP protein itself, the majority of the activity was Ca^{2+}-dependent. The IC_{50} values obtained for Ca^{2+}-dependent and -independent PKC activity, in a midbrain cytosolic preparation, with each substrate are given in Table 3.6. The potency of H7 on Ca^{2+}-independent activity showed little variation, phosphorylation of all substrates being inhibited with IC_{50} values in the range 12-45 μM. However there was greater variation between different substrates for Ca^{2+}-dependent activity, H7 being highly potent on GS peptide phosphorylation (IC_{50} 4 ± 1 μM).

A Ca^{2+}-dependent PKC is highly sensitive to H7

Figure 3.3 shows the inhibition of PKC activity from rat midbrain evoked by PDBu (a) and mezerein (b). While Ca^{2+}-dependent and -independent PDBu-evoked activity were inhibited with similar potencies (IC_{50} values 22 ± 1 and 28 ± 5 μM respectively), when activity was evoked by mezerein, the Ca^{2+}-dependent activity was potently inhibited by H7 (IC_{50} value 6 ± 1μM) compared with the Ca^{2+}-independent activity (IC_{50} value 28 ± 4 μM)(Figure 3.3b). However the Ca^{2+}-dependent activity represented a smaller proportion of the total activity evoked by mezerein (19%) than with PDBu (40%), even though the total amount of activity detectable was similar. All activity evoked by both of these activators was sensitive to inhibition by the more PKC selective inhibitor Ro 31-8220 (IC_{50} values 0.12-0.21 μM).

Potent inhibition by H7 of Ca^{2+}-dependent PKC activity was not only detected in rat midbrain, as shown in Table 3.4, as Ca^{2+}-dependent activity evoked by mezerein in cytosol from thalamus, spleen and lung was also highly sensitive to H7. As this potent inhibition was seen in all the tissues analysed using mezerein, it is possible that it could be a result of a direct interaction between mezerein and H7. However this seems unlikely as there was little difference in the potency of H7 on mezerein and PDBu-evoked Ca^{2+}-independent activity (Tables 3.3 and 3.4). Furthermore activation of this apparently highly H7-sensitive PKC was not specific to mezerein, as it could also be detected in midbrain cytosol when activity was induced by PDBu, using GS peptide as the substrate for the reaction (IC_{50} values for Ca^{2+}-dependent and Ca^{2+}-independent activity 4 ± 0.5 and 13 ± 2 μM respectively)(Figure 3.4, Table 3.6). There was also some evidence of highly H7-sensitive activity with MBP as the substrate (IC_{50} value in thalamus 6 ± 5 μM, Table 3.5), although this represented only a small proportion of the total activity in this tissue (12%) and highly H7-sensitive activity was not detected

in other tissues with this substrate. While both thalamus and midbrain contain all of the cPKCs, spleen and lung contain only PKC α and β, as PKC γ expression is restricted to the CNS. Thus this suggests that either PKC α or β may be responsible for this highly H7-sensitive PKC activity. To further investigate this possibility, both these isoforms were purified from rat brain by hydroxyapatite (HAP) chromatography. The PDBu-induced phosphorylation of GS peptide was measured, as there is more detectable activity with this substrate than with histone IIIS. H7 inhibited the Ca^{2+}-dependent activity in the PKC α fraction with an IC_{50} value of 31 ± 9 μM while the PKC β fraction was potently inhibited (IC_{50} value of 3 ± 1 μM)(Figure 3.5).

Characterisation of an H7-resistant form of PKC in anterior pituitary

The potency of H7 on PKC activity in anterior pituitary cytosol evoked by either PDBu (1 μM), mezerein (1 μM) or DOG (1 mM) was compared, using histone IIIS as the substrate, as these activators have shown differences in their effects on Ca^{2+} channels in pituitary cells (Mitchell et al., 1992). At these concentrations both Ca^{2+}-dependent and -independent PKC activity was almost maximal (M Johnson and R Mitchell; unpublished data). For all three activators, the Ca^{2+}-independent activity was relatively insensitive to H7 (IC_{50} values 145 ± 38, 148 ± 21 and 118 ± 42 μM respectively)(Figure 3.6). The Ca^{2+}-dependent activity induced by PDBu and DOG in anterior pituitary cytosol was H7 sensitive (IC_{50} values 25 ± 4, 21 ± 10 μM) and represented 43% and 53% respectively of the total PKC activity detected with each activator. As previously mentioned, 1 μM mezerein evoked maximal activity in the absence of Ca^{2+}.

As histone IIIS is reported to be a poor substrate for the Ca^{2+}-independent PKC isoforms (Schaap and Parker, 1990; Schaap et al., 1989),

and there is relatively little activity in anterior pituitary compared to other tissues, the PDBu-evoked activity in anterior pituitary cytosol was compared using a number of protein and synthetic peptide substrates. As shown in Table 3.7, when histone was replaced with either MBP or the α, ε or ζ pseudosubstrate peptides, there was little difference between the potency of H7 on the Ca^{2+}-independent and -dependent activity, with IC_{50} values in the range 20-40 μM. Thus H7-resistant activity was not detectable in anterior pituitary cytosol with these substrates. However, when GS peptide was used as the substrate, Ca^{2+}-independent PDBu-evoked activity was inhibited in a biphasic manner with the majority of the activity (55%) being relatively insensitive to H7 (IC_{50} value 84 ± 18 μM), the other 45% being potently inhibited by H7 (IC_{50} value 4 ±1). Therefore, as the activity detected using this substrate was higher than with histone IIIS, this substrate was used for subsequent purification studies.

3.4 Discussion

In this chapter, PKC activity partially purified from several tissue and cell line sources has been compared. The potency of PDBu on PKC from pituitary, midbrain and spleen, as well as the COS 7 monkey fibroblast cell line, differed considerably (Table 3.1), although in all cases PDBu was more potent in the presence of Ca^{2+}. This is consistent with previous evidence that PDBu binds with higher affinity to the Ca^{2+}-dependent PKCs than to the Ca^{2+}-independent isoforms (Kazanietz et al., 1993). Phorbol dibutyrate was least potent on PKC from COS 7 cells in the absence of Ca^{2+}, although in the presence of Ca^{2+}, the EC_{50} value was similar to those obtained for PKC from the other tissues. As these cells contain only PKC α and ζ (Kosaka et al., 1988; Ways et al., 1992) and PKC ζ is reported to be insensitive to phorbol esters (McGlynn et al., 1992; Nakanishi and Exton, 1992), this is

likely to reflect activation of PKC α in the presence and absence of Ca^{2+}. This difference in potency is in contrast to a previous report, which found that this isoform, produced in a baculovirus expression system, bound PDBu with similar affinity whether Ca^{2+} was present or not (Kazanietz et al., 1993). While the binding affinity may not be the only factor influencing the potency of activation by phorbol esters, it is also possible that recombinant PKC isoform may lack post-translational modifications, such as phosphorylation, that occur in mammalian cells (Borner et al., 1989; Pears et al., 1992). Indeed PKC isoforms produced in baculovirus expression in some cases have been found to display severely diminished activity (Rankl et al., 1994). There were also differences between tissues in the proportion of the total activity that was activated in the absence of Ca^{2+}, which is likely to reflect differences in the nPKC content of these tissues, as well as the extent to which the cPKCs present may be activated independently of Ca^{2+}. In COS 7 cells, which contain no nPKC isoforms, approximately 50% of the total activity was activated in the absence of Ca^{2+}. This is consistent with previous evidence that PKC α may be activated by phorbol esters in the absence of Ca^{2+} (Ryves et al., 1991).

Inhibition studies compared the effects of three catalytic domain inhibitors, staurosporine, H7 and Ro31-8220, and have shown differences in their inhibition properties. These compounds have all been reported to be ATP-competitive inhibitors (Davis et al., 1989; Hidaka et al., 1984; Tamaoki et al., 1986) but there is evidence to suggest that, although they all bind close to the ATP site, the precise sites of action appear to be different. H7 has been shown to compete kinetically with ATP (Hidaka et al., 1984) but only partially protects the ATP site against denaturation by covalent chemical reagents (Ohta et al., 1988), and this inhibitor displaces the binding of Ro 31-8220-sensitive [^{3}H]N,N-dimethyl staurosporine only at high concentrations

(Thomson et al., 1991). Furthermore it has been suggested that the potency with which staurosporine is reported to act in cells is too high for an purely ATP-competitive mechanism (Rüegg and Burgess, 1989).

In this study, staurosporine was equipotent on the Ca^{2+}-dependent and -independent activity from midbrain, pituitary and COS 7 cells. This compound is a potent PKC inhibitor but lacks selectivity, also inhibiting other serine/threonine kinases as well as some tyrosine kinases (Rüegg and Burgess, 1989). The more selective PKC inhibitor, Ro 31-8220 (Davis et al., 1989), has been reported to show a modest difference in potency between PKC isoforms (Nixon, 1992) and in this study there were small differences in potency of this inhibitor on PKC activity from the sources tested. The only marked differences in potency, however, occurred with H7; the Ca^{2+}-independent activity from anterior pituitary being particularly insensitive to this inhibitor (Table 3.2). This is consistent with previous reports from this laboratory that a number of pituitary cell functions are mediated by an H7-resistant form of PKC (Johnson et al., 1992b, MacEwan et al., 1992; Thomson et al., 1993b), and from other laboratories describing phorbol ester-evoked responses that are inhibited by staurosporine but not H7 (Nakadate et al., 1989; Watson et al., 1988). When the IC_{50} values for H7 were determined for a number of different tissues, there was little variation in potency on the Ca^{2+}-dependent activity from all the tissues tested including COS 7 cells where PKC α is the only phorbol ester-responsive isoform present)(Kosaka et al., 1988), spleen (containing some α but predominantly β of the cPKCs)(Shearman et al., 1987) and cerebellum (particularly rich in cPKC γ)(Shearman et al., 1987). This is consistent with previous evidence that the cPKCs are all sensitive to H7 (Pelosin et al., 1990). The IC_{50} values for Ca^{2+}-independent activity, however, showed wide differences, with pituitary and perhaps lung (but not the other sources tested) containing H7-

resistant activity (Table 3.3). Pituitary cells have been shown by immunoblotting to contain PKC α, β, δ, θ, ε and ζ but not the γ or η isoforms ((MacEwan, 1993) and D J MacEwan; unpublished data) while lung has been reported to contain the nPKCs δ and η but not ε, as well as PKC ζ (Bacher et al., 1991; Wetsel et al., 1992). However, Ca^{2+}-independent activity from thalamus and spleen, both containing large amounts of PKC δ (Leibersperger et al., 1990; Scott-Young, 1989) showed no evidence of H7-resistant activity (Table 3.3), and this isoform, purified from 3Y1 cells has been reported to be sensitive to this inhibitor (Uchida et al., 1991). PKC ε, though present in pituitary, is absent from lung (Wetsel et al., 1992) and has previously been shown to be H7-sensitive (Schaap and Parker, 1990). Furthermore, there is evidence that PKC ε is not able to efficiently phosphorylate histone (Schaap and Parker, 1990; Schaap et al., 1989). The other isoform common to both tissues, PKC ζ is reported not to be activated by phorbol esters (McGlynn et al., 1992; Nakanishi and Exton, 1992) and is also present in COS 7 cells (Ways et al., 1992), which showed no PDBu-evoked Ca^{2+}-independent activity. Thus the tissue distribution of this H7-resistant PDBu-evoked PKC activity clearly does not correspond to any of these nPKC or aPKC isoforms.

The replacement of PDBu with mezerein as the agonist to evoke PKC activity showed no evidence for any selectivity of this activator for the H7-resistant kinase (Table 3.4). It had been suggested that mezerein may be a good activator of the H7-resistant PKC in anterior pituitary as this activator had facilitatory effects on $^{45}Ca^{2+}$ influx through L-type Ca^{2+} channels in both anterior pituitary and GH_3 cells, a response that was resistant to H7 (Mitchell et al., 1992). However the results shown in Table 3.4 suggest that it may instead be a relatively poor activator of the PKC responsible for the inhibition of influx through these channels that is predominant when PDBu is the

activator. Thus, in a partially-purified cytosolic preparation containing essentially all the isoforms of PKC present in each tissue, it may show no clear selectivity for the H7-resistant PKC.

As histone is reported to be a poor substrate for the Ca^{2+}-independent PKC isoforms (Schaap and Parker, 1990; Schaap et al., 1989), MBP thiophosphorylation was also compared in a number of tissues. While there was little difference in the IC_{50} values for H7 inhibition of PKC activity for most tissues with either of these substrates, there was a clear difference in anterior pituitary (Table 3.5). In this tissue, the Ca^{2+}-independent activity was H7-resistant with histone IIIS but not MBP as the substrate. This does not necessarily mean that the H7-resistant kinase is unable to phosphorylate MBP, however, as if the activity of this form of PKC was similar with the two substrates, it is possible that it is masked by the activity of the other Ca^{2+}-independent isoforms present in anterior pituitary cytosol, which prefer MBP to histone IIIS as the substrate. The anterior pituitary contains the nPKCs δ and ε, both of which are reported to show greater activity towards this substrate that towards histone, with MBP being a particularly good substrate for PKC δ (Kazanietz et al., 1993).

In view of the differences evident when histone was replaced with MBP, and reports that cofactor-dependence varies with substrate (Saido et al., 1992), the properties of PKC from rat midbrain were compared with a variety of substrates. Differences were seen in both potency of H7 and Ca^{2+} dependence of the activity with each substrate (Table 3.6, Figure 3.2). Phosphorylation of the $AcMBP_{(4-14)}$ peptide, for example, was almost totally Ca^{2+}-dependent. It is possible that Ca^{2+} may interact directly with this peptide, to present it in a better conformation for phosphorylation, as it has been previously reported that Ca^{2+} enhances the activity of both cPKCs and nPKCs on this substrate (Saido et al., 1992). It is also possible that different

substrates may shift the position of the PDBu activation curve so the ability of 1 μM PDBu to activate different PKC isoforms may vary, affecting the Ca^{2+} dependence of evoked activity. There is evidence to suggest that some nPKCs may be inhibited by Ca^{2+} (Schaap and Parker, 1990) and that this phenomenon is substrate dependent (Saido et al., 1992). It is clear therefore that the proportions of Ca^{2+}-dependent and -independent activity in midbrain cytosol with each substrate is a result of a variety of different effects, each isoform present making a variable contribution to activity in the presence and absence of Ca^{2+}. The rate of phosphorylation of different substrates by individual isoforms varies considerably (Olivier and Parker, 1991), so the range of potencies for H7 inhibition are likely to reflect the differing contribution of each isoform to the total PKC activity, both Ca^{2+}-dependent and Ca^{2+}-independent.

Comparisons of the effect of H7 on PDBu and mezerein-evoked activity in a variety of tissues showed that, when mezerein was the activator, a form of Ca^{2+}-dependent PKC was highly sensitive to this inhibitor (Tables 3.3 and 3.4). While this could be due to some interaction of mezerein with H7, there was no difference in the potency H7 on mezerein and PDBu-evoked Ca^{2+}-independent activity. Phorbol esters and diterpenes, such as mezerein and PDBu interact with the C1 region of the regulatory domain (Burns and Bell, 1991) while the site of H7 action is in the catalytic domain of the enzyme (Hidaka et al., 1984). While there is one report that the potency of PKC inhibitors varies with the activator, this was only the case for inhibitors acting on the regulatory domain, while catalytic domain inhibitors such as H7 and staurosporine were equipotent regardless of the activator used (Robinson, 1992). Furthermore PDBu was also able to activate this form of PKC, as was evident when GS peptide was used as the substrate for midbrain PKC, as H7 was highly potent on the Ca^{2+}-dependent activity

detected in these conditions (Table 3.6, Figure 3.4). The presence of this potently H7-inhibited activity in both brain-derived cytosol and that from other tissues suggests that either PKC α or β may be responsible as PKC γ is expressed exclusively in the CNS (Shearman et al., 1987). As PDBu-evoked activity in partially-purified cytosol from COS 7 cells, which contain only cPKC α (Kosaka et al., 1988), showed no evidence of this highly H7-sensitive cPKC (Table 3.2), this suggested that PKC β may be responsible for this activity. GS peptide is reported to be a particularly good substrate for PKC β while it is not very efficiently phosphorylated by PKC α and γ (Kazanietz et al., 1993). Therefore PKC β is likely to make a larger contribution to the Ca^{2+}-dependent activity relative to the other cPKCs when GS peptide is the substrate, which could be the reason why highly H7-sensitive activity was detectable using this substrate. This highly H7-sensitive PKC also appears to be able to make a contribution towards the Ca^{2+}-independent activity, as a component of Ca^{2+}-independent GS peptide thiophosphorylation in anterior pituitary was potently inhibited by H7 (Table 3.7). Subsequent analysis of PKC α and β purified from rat brain by HAP chromatography, which showed that Ca^{2+}-dependent activity in the PKC β but not the PKC α fraction was highly sensitive to H7 (Figure 3.5). Differences in sensitivity to inhibition by H7 have previously been detected in physiological models of PKC mediated processes. Phorbol dibutyrate inhibits depolarisation-induced Ca^{2+} influx through L-type Ca^{2+} channels in the anterior pituitary-derived GH_3 cell line and this is highly sensitive to inhibition by H7 (IC_{50} value 7 ± 4 μM)(Mitchell et al., 1992). PDBu-evoked LH secretion from the anterior pituitary has a component that is highly H7-sensitive (Thomson et al., 1993b). These *in vitro* studies suggest that this may be due to the involvement of PKC β, which is consistent with evidence

that the addition of either PKC α or β to permeabilised pituitary cells in primary cultures causes LH secretion (Naor et al., 1989).

As previously mentioned, PKC activators have been shown to elicit quite different effects on $^{45}Ca^{2+}$ influx through L-type Ca^{2+} channels in the anterior pituitary compared to the GH_3 cell line (MacEwan and Mitchell, 1991; MacEwan et al., 1991), and to differ in their ability to activate individual PKC isoforms *in vitro* (Ryves et al., 1991). This study has shown that both mezerein and DOG, in addition to PDBu, were able to evoke H7-insensitive Ca^{2+}-independent PKC activity in pituitary cytosol (Figure 3.6), consistent with the ability of these compounds to activate an H7-resistant form of PKC which facilitates Ca^{2+} entry through L-type channels (Johnson et al., 1993; Mitchell et al., 1992). It has been reported that certain diterpenes are capable of activating cPKCs in the absence of Ca^{2+} (Ryves et al., 1991) but there is evidence that DOG is only able to activate PKC α and possibly β in the presence of Ca^{2+} (Johnson et al., 1992a, Johnson et al., 1994). Thus the ability of DOG to evoke the H7-resistant kinase activity suggests that this is not due to Ca^{2+}-independent cPKC activity.

Thus the evidence presented in this chapter suggests that the H7-insensitive kinase, found in pituitary and apparently to a lesser extent in lung, does not correspond to any of the well-characterised Ca^{2+}-independent isoforms, and it is not a cPKC activated in the absence of Ca^{2+}. This activity may represent one of the incompletely characterised isoforms, such as PKC θ or λ; alternatively it may be a modified form of one of the known PKCs or perhaps a novel PKC isoform.

Table 3.1 Activation by PDBu of PKC activity partially purified cytosol from the cytosolic fraction of various tissues and cell lines.

Tissue	Activity in the absence of Ca^{2+} (% of total activity)	EC_{50} value (μM)	
		- Ca^{2+}	+ Ca^{2+}
pituitary	75 ± 6	0.73 ± 0.33	0.43 ± 0.24
midbrain	62 ± 4	0.88 ± 0.13	0.10 ± 0.03
spleen	80 ± 7	0.62 ± 0.12	0.08 ± 0.01
COS 7	48*	2.3 ± 1.3	0.15 ± 0.07

Phosphatidylserine-dependent histone IIIS thiophosphorylation was elicted by seven concentrations of PDBu (0-3 μM) in the absence (<3 nM) or presence (100 μM free) of Ca^{2+}. The EC_{50} values were determined by non-linear curve fitting (P. Fit) as descibed in Section 3.2. Values are means ± S.E.M. ($4 \leq n \leq 6$).

* With COS 7 cells, the PKC activity curve did not reach a clear plateau, therefore a mean maximum % activity figure was assigned, on the basis of the response at the highest concentration tested (10 μM). This putative value was used in the computer curve fitting to determine a hypothetical EC_{50} value.

Table 3.2 Effects of H7, staurosporine and Ro 31-8220 on PDBu-evoked PKC activity partially purified from the cytosolic fraction of midbrain, anterior pituitary and COS 7 cells.

Tissue		IC_{50} (μM)		
		H7	staurosporine	Ro31-8220
midbrain	Ca^{2+}-independent	28 ± 5	0.12 ± 0.01	0.18 ± 0.03
	Ca^{2+}-dependent	22 ± 1	0.10 ± 0.04	0.19 ± 0.01
pituitary	Ca^{2+}-independent	145 ± 38	0.10 ± 0.04	0.34 ± 0.07
	Ca^{2+}-dependent	25 ± 4	0.12 ± 0.05	0.14 ± 0.03
COS 7 cells	Ca^{2+}-dependent	36 ± 11	0.17 ± 0.03	0.26 ± 0.05

Phosphatidylserine-dependent histone IIIS thiophosphorylation was evoked by 1 μM PDBu at various concentrations of H7 (1-300 μM), staurosporine (0.01-1 μM) and Ro 31-8220 (0.01-30 μM) in the absence (<3 nM) or presence (100 μM free) of Ca^{2+}. The IC_{50} value determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$). There was no detectable Ca^{2+}-independent activity in COS 7 cells.

Table 3.3 Effect of H7 on PDBu-evoked PKC activity partially purified from the cytosolic fraction of various tissues and cell lines.

Region	IC_{50} (μM)	
	Ca^{2+}-independent activity	Ca^{2+}-dependent activity
pituitary	145 ± 38	25 ± 4
sheep pituitary	128 ± 13	N.D.
lung	87 ± 37	39 ± 6
midbrain	28 ± 5	22 ± 1
thalamus	43 ± 7	23 ± 4
cerebellum	38 ± 15	34 ± 9
spleen	19 ± 3	25 ± 3
intestine	23 ± 4	N.D.
liver	15 ± 11	N.D.
COS 7 cells	N.D.	36 ± 11

Phosphatidylserine-dependent histone IIIS thiophosphorylation was evoked by 1 μM PDBu at six concentrations of H7 (0.01-300 μM) in the absence (<3 nM) or presence (100 μM free) of Ca^{2+}. The IC_{50} values were determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D. not detectable.

Table 3.4 Effect of H7 on mezerein-evoked PKC activity partially purified from the cytosolic fraction of various tissues.

Region	IC_{50} (μM)	
	Ca^{2+}-independent activity	Ca^{2+}-dependent activity
pituitary	148 ± 21	N.D.
lung	75 ± 10	3 ± 1
midbrain	28 ± 4	6 ± 1
thalamus	37 ± 8	6 ± 1
spleen	31 ± 5	7 ± 1

Phosphatidylserine-dependent histone IIIS thiophosphorylation was evoked by 1 μM mezerein at six concentrations of H7 (0.01-300 μM) in the absence (<3 nM) or presence (100 μM free) of Ca^{2+}. The IC_{50} value determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D. not detectable.

Table 3.5 Effect of H7 on PDBu-evoked PKC activity using myelin basic protein as the substrate.

Region	IC_{50} (μM)	
	Ca^{2+}-independent activity	Ca^{2+}-dependent activity
pituitary	34 ± 7	33 ± 4
midbrain	43 ± 6	40 ± 3
intestine	36 ± 1	N.D.
thalamus	37 ± 2	6 ± 5
spleen	27 ± 2	31 ± 8
skin	150 ± 30	N.D.

Phosphatidylserine-dependent MBP thiophosphorylation was evoked by 1 μM PDBu at six concentrations of H7 (0.01-300 μM) in the absence (<3 nM) or presence (100 μM free) of Ca^{2+}. The IC_{50} value determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D. not detectable.

Table 3.6 Effect of H7 on PKC activity in partially-purified cytosol from rat midbrain with various substrates.

Substrate	IC_{50} (μM)	
	Ca^{2+}-independent activity	Ca^{2+}-dependent activity
Histone IIIS	28 ± 5	22 ± 1
Myelin Basic Protein (MBP)	43 ± 6	40 ± 3
$AcMBP_{(4-14)}$ peptide	12 ± 5	25 ± 7
α peptide	28 ± 9	26 ± 9
ε peptide	34 ± 1	17 ± 0.3
GS peptide	13 ± 2	4 ± 0.5

Phosphatidylserine-dependent thiophosphorylation was evoked by 1 μM PDBu at six concentrations of H7 (0.01-300 μM) in the absence (<3 nM) or presence (100 μM free) of Ca^{2+}. The IC_{50} value determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D. not detectable. Substrate concentrations: proteins, 1.25 mg/ml; ε pseudosubstrate peptide, 20 μM; $AcMBP_{(4-14)}$ peptide, 50 μM; GS peptide, 100 μM.

Table 3.7 Effects of H7 on PDBu-evoked anterior pituitary PKC activity with various substrates

Substrate	IC_{50} value (μM)
Histone IIIS	145 ± 38
Myelin Basic Protein (MBP)	34 ± 7
α peptide	32 ± 3
ε peptide	40 ± 5
ζ peptide	22 ± 8
GS peptide	4 ± 1 45%* 84 ± 18 55%

Phosphatidylserine-dependent thiophosphorylation was evoked by 1 μM PDBu at six concentrations of H7 (0.01-300 μM) in the absence (<3 nM) or presence (100 μM free) of Ca^{2+}. The IC_{50} value determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D. not detectable. Substrate concentrations: proteins, 1.25 mg/ml; ε pseudosubstrate peptide, 20 μM; $AcMBP_{(4-14)}$ peptide, 50 μM; GS peptide, 100 μM.

* When GS peptide was used as the substrate, Ca^{2+}-independent PDBu-evoked activity was inhibited in a biphasic manner with the majority of the activity (55%) being relatively insensitive to H7, the other 45% being potently inhibited by H7.

Figure 3.1 Activation by PDBu of partially-purified cytosolic PKC activity from rat anterior pituitary.

Histone IIIS thiophosphorylation was measured at various concentrations of 4 β-PDBu (0.3-10 μM) both in the presence and absence of Ca^{2+} (100 μM and <3 nM free Ca^{2+} respectively). All points are means ± S.E.M. ($4 \leq n \leq 6$). Baseline activity with PS alone was subtracted from activator curves and the PDBu-evoked activity was normalised to the activity obtained with the maximally effective dose (10 μM, in the presence of 100 μM Ca^{2+}). Curve fitting was then conducted as described in the Section 3.2 and the EC_{50} values obtained were 637 ± 311 nM and 380 ± 117 nM in the absence and presence of Ca^{2+} respectively. The specificity of the reaction was tested by replacing 4-β PDBu with the essentially inactive stereoisomer, 4-α PDBu. (●) 4-β PDBu in the presence of Ca^{2+}, (■) 4-β PDBu in the absence of Ca^{2+}, (▲) 4-α PDBu in the presence of Ca^{2+}.

Figure 3.1

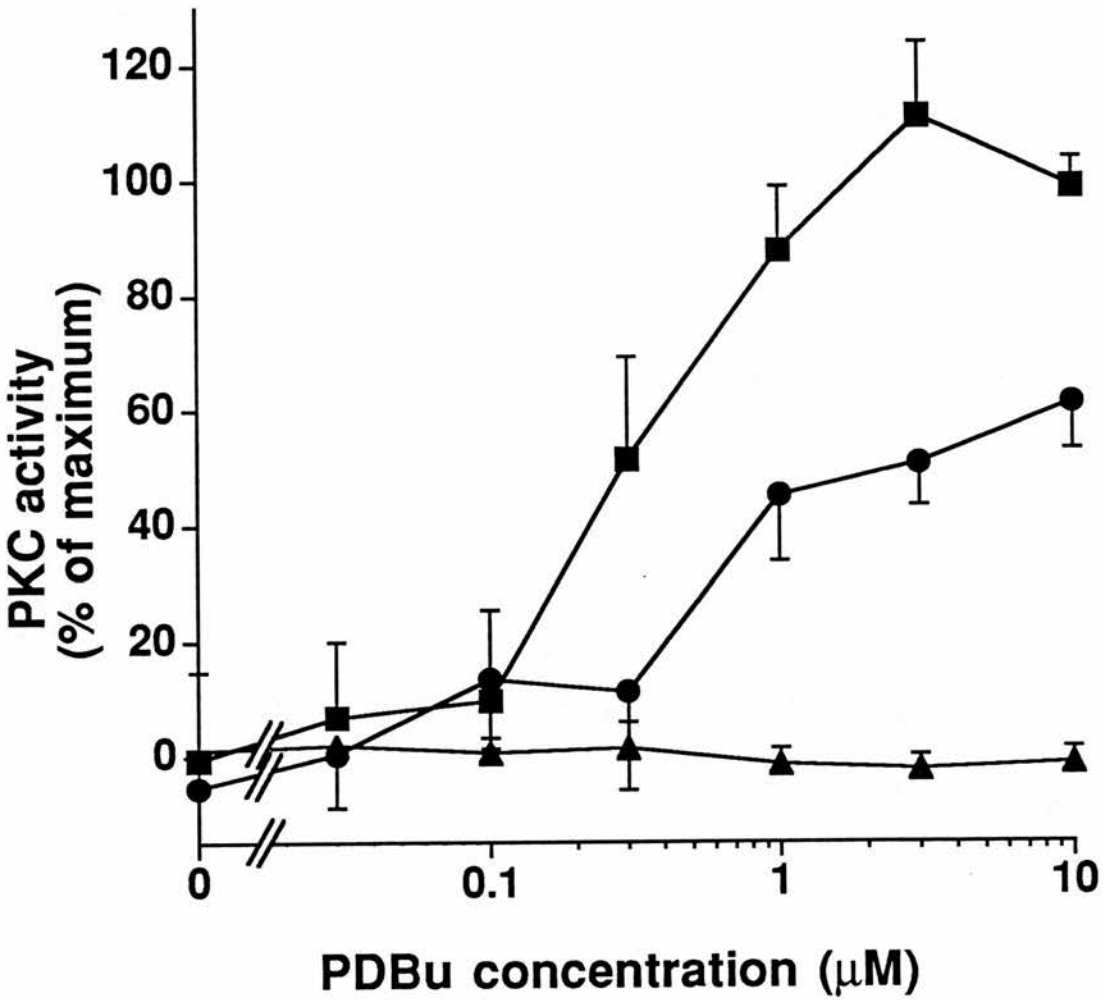

120
100
80
60
40
20
0
PKC activity
(% of maximum)
0
0.1
1
10
PDBu concentration (μM)

Figure 3.2 Comparison of PDBu-evoked thiophosphorylation of various protein and peptide substrates.

Phorbol dibutyrate-evoked thiophosphorylation of protein and peptide substrates was compared in both the absence and presence of Ca^{2+} (<3 nM and 100 μM respectively). Activity was normalised to the amount of histone IIIS thiophosphorylation measured in the presence of Ca^{2+}. The sequences of the peptide substrates used are as follows: α peptide (RFARKGSLRQKNV), ε peptide (ERMRPRKRQGSVRRRV), GS peptide (PLSRTLSVAAKK), $MBP_{(4-14)}$ peptide (AcQKRPSQRSKYL) and histone H1 phosphorylation site peptide (RRKASGP).

Figure 3.2

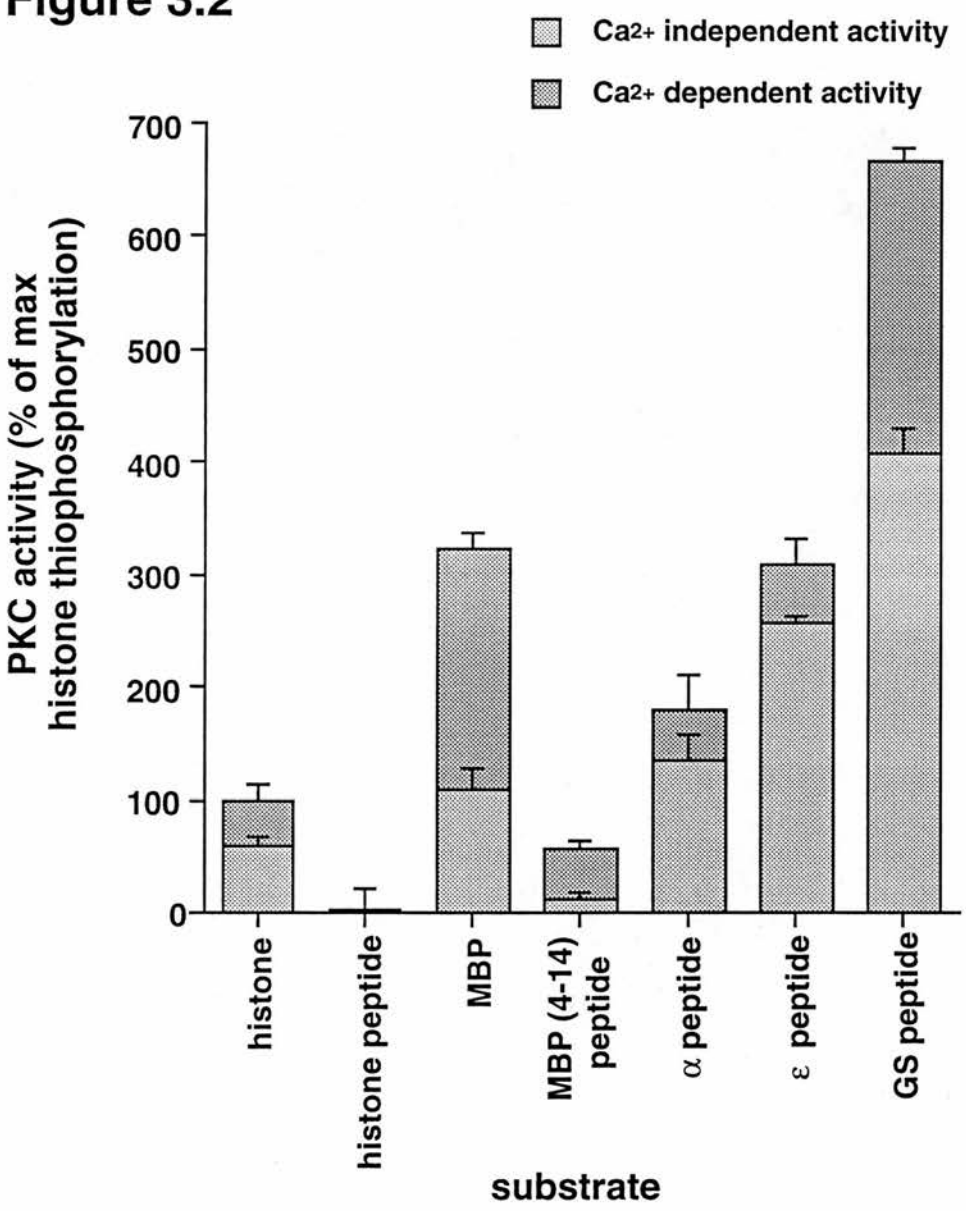

Ca2+ independent activity
Ca2+ dependent activity
PKC activity (% of max histone thiophosphorylation)
700
600
500
400
300
200
100
0
histone
histone peptide
MBP
MBP (4-14) peptide
α peptide
ε peptide
GS peptide
substrate

Figure 3.3 Inhibition by H7 of PKC activity in rat midbrain with different activators.

Histone IIIS thiophosphorylation evoked by (a) PDBu (1 μM) or (b) mezerein (1 μM) was measured at various concentrations of H7 in the presence or absence of Ca^{2+} (<3 nM or 100μM respectively). All points are mean ± S.E.M. ($4 \leq n \leq 6$). IC_{50} values for Ca^{2+}-independent activity evoked by PDBu and mezerein were 28 ± 5 and 28 ± 4 μM respectively. Ca^{2+}-independent activity was subtracted from the activity in the presence of Ca^{2+} as described in Section 2.6 and the IC_{50} values obtained for PDBu and mezerein-evoked activity were 22 ± 1 and 6 ± 1 respectively. All activity was sensitive to inhibition by Ro 31-8220 with IC_{50} values for PDBu of 180 ± 30 and 190 ± 10 nM and for mezerein of 210 ± 30 and 120 ± 10 nM for Ca^{2+}-independent and -dependent activity respectively. (●) Ca^{2+}-independent, (■) Ca^{2+}-dependent activity.

Figure 3.3

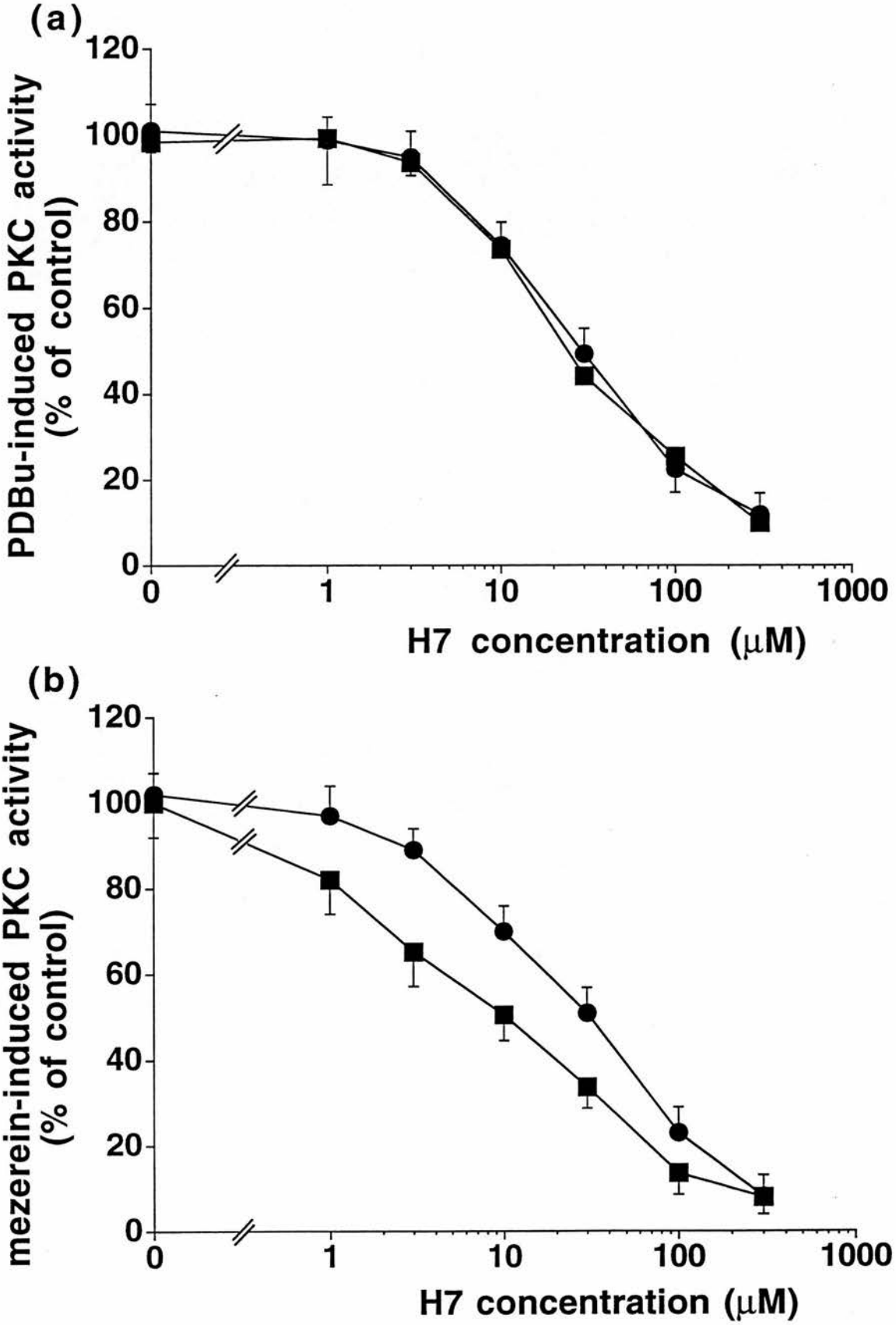

(a)
PDBu-induced PKC activity
(% of control)
120
100
80
60
40
20
0
0
1
10
100
1000
H7 concentration (μM)
(b)
mezerein-induced PKC activity
(% of control)
120
100
80
60
40
20
0
0
1
10
100
1000
H7 concentration (μM)

Figure 3.4 Inhibition by H7 of PKC activity from rat midbrain. Phorbol 12, 13-dibutyrate (1 μM)-evoked PKC activity partially-purified from the cytosolic fraction of rat midbrain was measured using GS peptide (100 μM) as the substrate. The effect of various concentrations of H7 was determined in both the presence and absence of Ca^{2+} (100 μM and <3 nM free Ca^{2+} respectively). The IC_{50} values were determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. All points are means ± S.E.M. ($4 \leq n \leq 6$). IC_{50} values were 13 ± 2 and 4 ± 0.5 μM for Ca^{2+}-independent and Ca^{2+}-dependent activity respectively. (●) Ca^{2+}-independent, (■) Ca^{2+}-dependent activity.

Figure 3.4

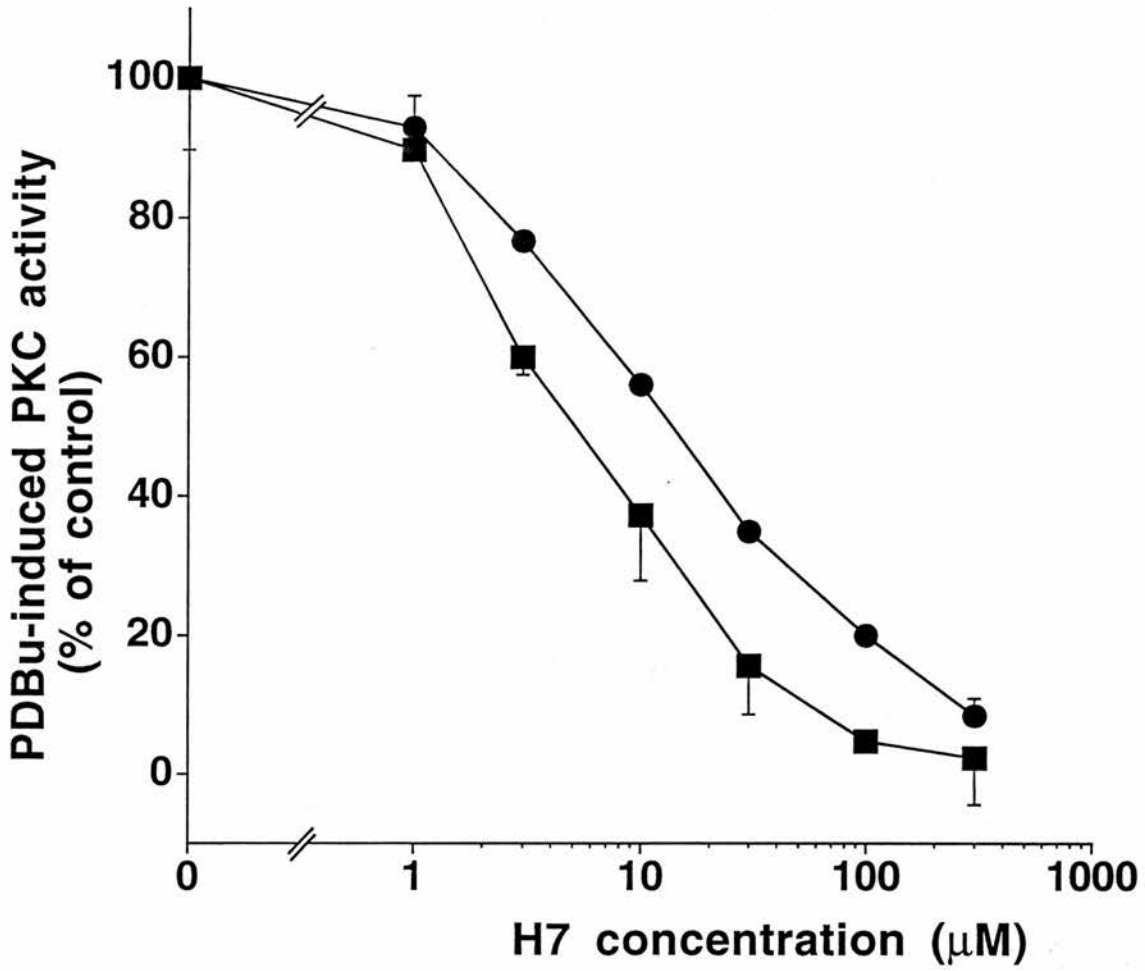

PDBu-induced PKC activity
(% of control)
100
80
60
40
20
0
0
1
10
100
1000
H7 concentration (μM)

Figure 3.5 Inhibition by H7 of PKC α and β purifed from rat brain by hydroxyapatite chromatography.

Phorbol 12, 13-dibutyrate (1 μM)-evoked PKC activity was measured using GS peptide (100 μM) as the substrate. The effect of various concentrations of H7 was determined both in the presence and absence of Ca^{2+} (100 μM and <3 nM free Ca^{2+} respectively) and Ca^{2+}-independent activity was subtracted from the activity in the presence of Ca^{2+} to obtain Ca^{2+}-dependent activity, as described in Section 2.6 . IC_{50} values for Ca^{2+}-dependent activity were 31 ± 9 and 3 ± 1 μM for PKC α and β respectively. All points are means ± S.E.M. ($4 \leq n \leq 6$). (●) PKC α, (■) PKC β.

Figure 3.5

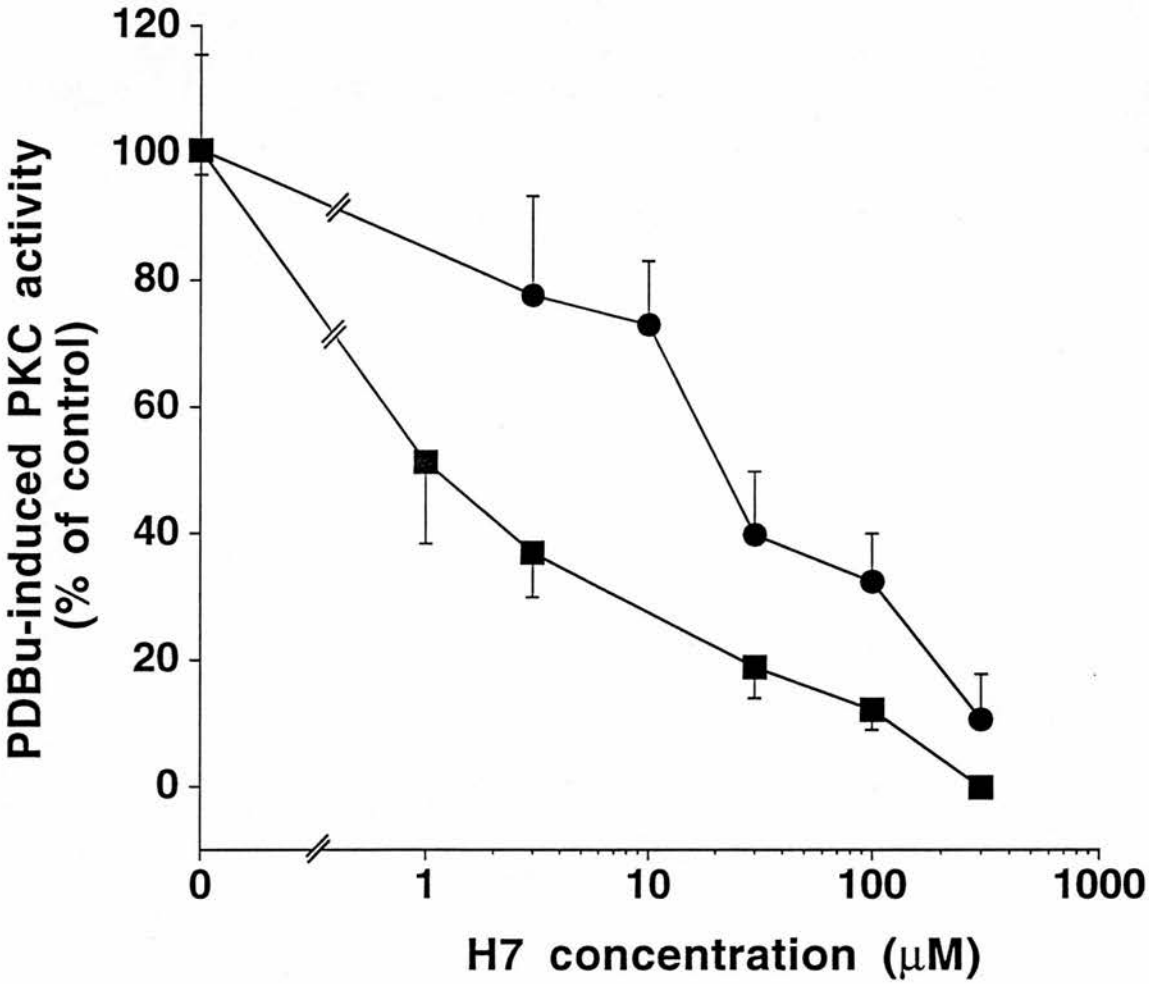
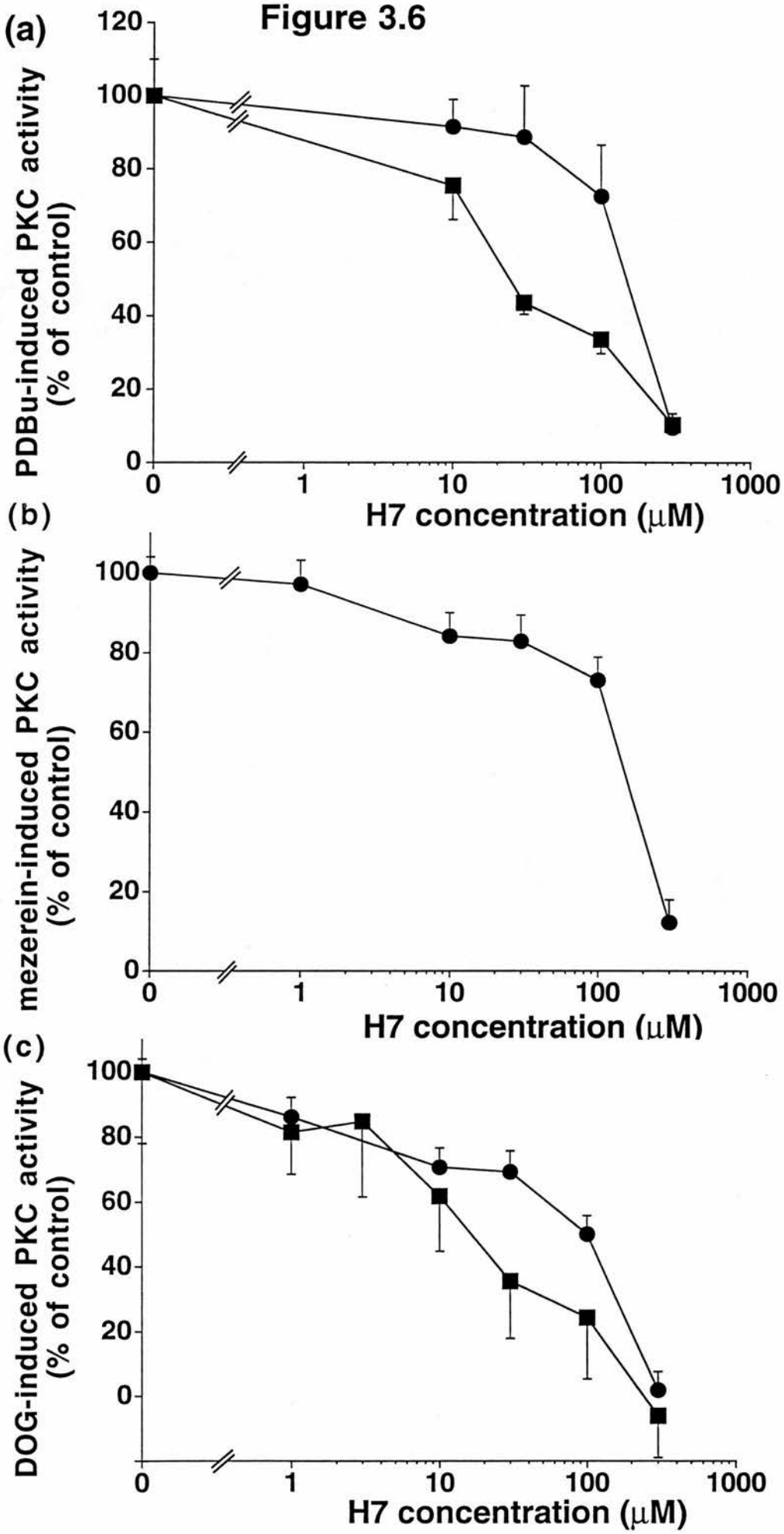

120
100
80
60
40
20
0
PDBu-induced PKC activity
(% of control)
0
1
10
100
1000
H7 concentration (μM)

Figure 3.6 Inhibition by H7 of anterior pituitary PKC activity evoked by different activators.

Histone IIIS thiophosphorylation evoked by (a) PDBu (1 μM), (b) mezerein (1 μM) or (c) DOG (1mM) was measured at various concentrations of H7 in the presence or absence of Ca^{2+} (<3 nM or 100μM respectively). All points are mean ± S.E.M. ($4 \leq n \leq 6$). IC_{50} values for Ca^{2+}-independent activity evoked by PDBu, mezerein or DOG were 145 ± 38, 148 ± 21 and 118 ± 42 μM respectively. Ca^{2+}-independent activity was subtracted from the activity in the presence of Ca^{2+} as described in Section 2.6 and the IC_{50} values obtained for PDBu or DOG -evoked activity were 25 ± 4 and 21 ± 10 μM respectively. Mezerein evoked no additional activity in the presence of Ca^{2+}. (●) Ca^{2+}-independent, (■) Ca^{2+}-dependent activity.

Figure 3.6


(a)
PDBu-induced PKC activity (% of control)
120
100
80
60
40
20
0
0
1
10
100
1000
H7 concentration (μM)
(b)
mezerein-induced PKC activity (% of control)
100
80
60
40
20
0
0
1
10
100
1000
H7 concentration (μM)
(c)
DOG-induced PKC activity (% of control)
100
80
60
40
20
0
0
1
10
100
1000
H7 concentration (μM)

CHAPTER 4

HYDROXYAPATITE PURIFICATION AND FURTHER CHARACTERISATION OF THE H7-RESISTANT PKC FROM ANTERIOR PITUITARY

4.1 INTRODUCTION

The identification of PKC as a Ca^{2+}-dependent protein kinase requiring phospholipid as a cofactor (Takai et al., 1979a), and reports of the widespread distribution of this kinase in various tissues and animal species (Kuo et al., 1980), soon lead to investigations into the biochemical properties of this enzyme. Initial characterisation of PKC activity was conducted on enzyme purified from a number of tissues sources, including brain, spleen and heart (Kikkawa et al., 1982; Schatzman et al., 1983; Wise et al., 1982). However, while purification of up to 15 000 fold was reported (Wise et al., 1982), in each case the procedure required several steps, the yield was low and the resulting enzyme preparation was highly unstable. A simplified procedure was developed by Uchida et al, which produced a yield of 30-50% while achieving up to 7700 fold purification, and PKC was purified by this method from renal cortex (Uchida and Filburn, 1984). This procedure involved DEAE cellulose chromatography followed by PS affinity chromatography, and this was the first report to suggest that other Ca^{2+}- and phospholipid-dependent protein kinases might be present in the resulting enzyme preparation. However, it was only with the introduction of hydroxyapatite (HAP) chromatography into the purification process that PKC was separated into different isoforms (Huang et al., 1986b). In this study, PKC from rat brain was purified using DEAE cellulose, followed phenyl sepharose, Sephacryl S-200 and polylysine agarose, but the different isoforms were found to copurify from all of these procedures, suggesting that they shared similar charge, size and affinity characteristics. Subsequent elution from a HAP column resulted in the now well-documented three peaks of Ca^{2+}-dependent PKC activity which

were designated as type I, II and III, and were later found to correspond to the isoforms encoded by the γ, β and α genes respectively (Kikkawa et al., 1987).

Since this initial report of the separation of isoforms from rat brain, additional members of the PKC family have been identified (Bacher et al., 1991; Baier et al., 1993; Ono et al., 1988; Pfizenmaier et al., 1993; Selbie et al., 1993). Some of these Ca^{2+}-independent PKCs have also been detected in brain (Selbie et al., 1993; Wetsel et al., 1992), so must be present within the three fractions originally called PKC types I-III. Indeed when extracts from rat retina were purified by HAP chromatography, PKC δ, ε and ζ immunoreactivity was detected within main three peaks of PKC activity (Fujisawa et al., 1992). Purification of individual PKC isoforms to homogeneity has been reported by a number of groups (Koide et al., 1992; Leibersperger et al., 1990; Nakanishi and Exton, 1992; Saido et al., 1992), each using multiple step procedures that are specifically designed to remove the other isoforms present in the tissues extract at particular stages.

The cloning of cDNAs corresponding to various PKC isoforms and subsequent expression of these cDNA, using either baculovirus expression systems or mammalian cell lines, has lead to the production of recombinant PKC isoforms. However this has not superseded the need to study native PKC purified from tissue sources. While in many cases the purified enzyme shows similar properties to the recombinant protein (Olivier et al., 1992), there are some exceptions. Protein kinase C δ, for example, when purified from porcine spleen showed reasonable activity when activity was measured with histone IIIS (~300 U/mg)(Leibersperger et al., 1990) while the recombinant enzyme was found to be a poor histone kinase (activity of 70 U/mg)(Olivier and Parker,

1991). Furthermore results obtained in this laboratory have shown differences in the potency of PKC activators on purified PKC isoforms (Johnson et al., 1994) that were not detected with recombinant PKC isoforms from a baculovirus expression system (Kazanietz et al., 1993). Indeed PKCs produced in such systems have been found to show reduced catalytic activity depending on the promoter used (Rankl et al., 1994). Such differences may reflect post-translational modifications such as phosphorylation (Connor and Clegg, 1993; Pears et al., 1992) and comparisons of native and expressed isoforms may provide important information about the regulation of these isoforms *in vivo*, in mammalian cells.

The results presented in Chapter 3 show that a form of PKC is present in anterior pituitary cytosol that may be distinguished by its relative resistance to the PKC inhibitor H7 (Table 3.1), and this PKC shows a tissue distribution that is distinct from that reported for any of the well-characterised PKC isoforms (Table 3.4). In order to further characterise the properties of this H7-resistant PKC, and to investigate whether it is a modified form one of the previously identified PKCs or whether it may represent a novel isoform of PKC, it was necessary to biochemically separate this PKC from other pituitary PKCs. Following initial studies using DEAE cellulose, fractionation of pituitary PKC activity was carried out using HAP chromatography, this method, as previously mentioned, being capable of partially resolving the individual PKC isoforms. In this Chapter, the elution profile of the H7-resistant PKC is compared with that of the other PKC isoforms present in the anterior pituitary, and evidence is presented to suggest that the H7-resistant kinase may represent a novel form of PKC.

4.2 Specific Methodology

DEAE cellulose separation

Anterior pituitaries from 20 male Wistar rats were homogenised and centrifuged as described in Section 2.4. The resulting cytosolic preparation was applied to a DEAE cellulose column at 4°C, the matrix was washed with 6 column volumes of homogenisation buffer, as described in Section 2.4 and the partially-purified PKC was eluted with 3 column volumes of buffers containing 50, 100, 150 and 200 mM NaCl sequentially. The Ca^{2+}-independent PKC activity in each eluate was then assayed and the effect of H7 determined.

Purification by hydroxyapatite chromatography

PKC isoforms were resolved by HAP chromatography as described in Section 2.5. Midbrain, lung or anterior pituitary tissue, from 6, 4 or 50 male Wistar rats respectively, was homogenised and PKCs were partially purified on DEAE cellulose before application to the HAP column. αT3-1 cells were grown in DMEM containing Na-pyruvate (0.11 g/l), penicillin/streptomycin (100 U/ml each) and 10% foetal calf serum. Confluent αt3-1 cells were then washed and harvested before homogenisation and DEAE cellulose chromatography. Proteins were eluted in a linear potassium phosphate gradient (5-300 mM; total volume 150 ml, fraction volume 2.5 ml) and the PKC activity was assayed. The resulting profile was assessed and, in most cases, fractions were pooled as appropriate for dialysis against 50% glycerol, 10 mM Tris HCl, 0.5 mM EDTA, 0.5 mM EGTA, 25 mM 2-mercaptoethanol (pH 7.5). Pooled fractions were stored at -20 °C. Alternatively, for the consensus PKC immunoblot on unpooled anterior pituitary fractions, 0.5 ml of every

second fraction was combined with 0.5 ml of the subsequent fraction for subsequent concentration and immunoblotting.

Mixed micelle PKC activity assay

Protein kinase C activity in partially purified from cytosol or in the more extensively purified HAP eluate was determined as the PDBu-evoked thiophosphorylation of GS peptide ([PLSRTLSVAAKK], modified from the sequence of residues 1-12 of glycogen synthase (House and Kemp, 1987)), measured in the presence of PS. The method used is described in detail in Section 2.4. Reactions were started by the addition of enzyme, incubated at 30°C for 15 min and stopped by addition of 20 µl TCA (6.8% w/v final). After incubation on ice for 15 min, TCA-precipitable material was removed by centrifugation and the supernatant was spotted onto 2x2 cm pieces of P-81 cellulose phosphate ion-exchange chromatography paper (Whatman International Ltd, Maidstone, Kent, UK), which were washed extensively in 75 mM H_3PO_4, dried and counted by liquid scintillation.

Immunoblotting with antibodies to PKC isoforms

Immunoblots were carried out as described in Section 2.7. Protein kinase C isoforms were identified with rabbit polyclonal antisera raised to isoform-specific peptide sequences in PKC α, δ, ε and ζ (2 µg/ml)(Gibco BRL, Paisley, Renfrew, UK) and β_1 (2.5 fold dilution)(Marais and Parker, 1989). The specificity of staining with these antisera was confirmed in each case using antibody blocked by preincubation with the relevant antigenic peptide (1 µg/ml). Protein kinase C θ was detected using a mouse monoclonal antibody (1 µg/ml; Transduction Labs, Lexington, KY, USA). Midbrain, pituitary and lung HAP fractions were also immunoblotted with a rabbit polyclonal consensus antibody, raised to a

sequence in the C4 domain conserved between all the PKC isoforms (PKC α:[Ac 543-550-Cys]) (Calbiochem). Unpooled pituitary HAP fractions combined in pairs were precipitated with TCA (final concentration 5% (w/v)) and deoxycholate was added to a final concentration of 0.0125% (w/v). Samples were then centrifuged (5 min, 4°C) and resulting pellet washed with cold acetone/water (9:1) before dissolving in SDS-PAGE buffer and electrophoresis and immunoblotting with the PKC concensus antibody as described in Section 2.7.

4.3 RESULTS

Protein kinase C activity in pituitary DEAE-cellulose eluates

Ca^{2+}-independent PKC activity from anterior pituitary tissue, measured with GS peptide as the substrate, was shown in Chapter 3 to be composed of 2 components which varied in their sensitivity to H7 (Table 3.6). To investigate the possibility of biochemically separating the H7-resistant PKC from other nPKCs, anterior pituitary cytosol was fractionated on a DEAE-cellulose column, by elution using buffers containing 50 mM, 100 mM, 150 mM and 200 mM NaCl sequentially. The Ca^{2+}-independent PDBu-evoked PKC activity in each fraction was measured and the majority (50%) was detected in the 50-100 mM fraction. The 0-50 and 100-150 mM fractions contained 33% and 12% of the total Ca^{2+}-independent PKC activity respectively, while the 150-200 mM fraction contained only 5% of the activity. The effect of H7 on the three fractions containing the most PDBu-evoked activity was measured and the IC_{50} values determined. Activity that was relatively resistant to H7 was detected only in the 100-150 mM NaCl fraction (IC_{50} 77 ± 11 μM); PKC activity in the other fractions was sensitive to this inhibitor (Table 4.1).

Hydroxyapatite fractionation of PKC activity from a variety of tissues and cell lines.

Figure 4.1 shows typical elution profiles of PKC activity from a) midbrain b) anterior pituitary and c) COS 7 cells when fractionated by HAP chromatography. Midbrain was selected as a control tissue for comparison with anterior pituitary as midbrain contains all of the well-characterised PKC isoforms (Scott-Young, 1989), but does not contain detectable quantities of the H7-resistant PKC (Figure 3.4). COS 7 cells were also compared as these cells also show no evidence of containing PDBu-evoked H7-resistant kinase activity (Table 3.1) and they are reported to contain only PKC α and perhaps ζ (Kosaka et al., 1988; Ways et al., 1992). Fractionation of midbrain extracts by HAP chromatography resulted in three main peaks of PKC activity as previously described for whole brain tissue (Huang et al., 1986b)(Figure 4.1a). A substantial proportion of the activity in the smallest, initial peak was phospholipid-independent and may represent cleaved catalytic subunits of PKC. The other two peaks of PKC activity were both of similar height, though the first of these eluted over more fractions than the second.

Hydroxyapatite chromatography of anterior pituitary extracts also revealed two main peaks of PS-dependent PKC activity (Figure 4.1b) although, in this case, the first one was much greater in magnitude than the second. However there was also a small peak of activity following the second main peak (fraction V) that was not present in midbrain or COS 7 cells (Figure 4.1a and c). Furthermore this was not seen in HAP profiles from most other tissues examined, for example cerebellum (R A Clegg, A J Ison and R Mitchell; unpublished work), spleen, and mammary gland (Connor and Clegg, 1993). Samples were pooled and five fractions were

collected from both pituitary and midbrain for further investigation, as shown in Figure 4.1.

The HAP elution profiles were also investigated for rat lung and the αT3-1 gonadotroph cell line, as both these sources are thought to contain the H7-resistant PKC (Table 3.3 and R Mitchell, R A Clegg and M S Johnson; unpublished data). As shown in Figure 4.2, HAP fractionation of extracts from both rat lung and αT3-1 cells resulted in profiles similar to that seen for anterior pituitary extracts, with some PKC activity eluting after the second main peak, although in both these cases a smaller proportion of the total activity was contained in the first main peak. Fractions of HAP column eluate from both lung and αT3-1 cells were also collected for further analysis.

The effect of H7 on PKC activity in pooled HAP fractions

The effect of H7 was examined on PDBu-evoked PKC activity in five pooled fractions of pituitary HAP eluate, selected to represent the main features of the HAP elution profile (Fractions I-V as shown in Figure 4.1). Fraction V was found to contain Ca^{2+}-independent activity that was relatively insensitive to this inhibitor (IC_{50} 85 ± 10 μM; Figure 4.3). Ca^{2+}-independent activity in fractions I-III was sensitive to H7 (IC_{50} values in the range 9-18 μM), while an intermediate IC_{50} value (45 ± 19 μM) was obtained for fraction IV (Table 4.2). Ca^{2+}-dependent activity was not detected in fraction II but was present in all other fractions and in each case it was H7-sensitive (IC_{50} values in the range 10-23 μM)(Table 4.2). All PDBu-evoked activity was sensitive to inhibition by the selective PKC inhibitor Ro 31-8220, with IC_{50} values for all the Ca^{2+}-independent activity in fractions I-V in the range 103-169 nM. The Ca^{2+}-independent PKC activity in the corresponding fractions from midbrain was also

examined and the effect of H7 determined. As seen in Table 4.3, all fractions were sensitive to this inhibitor with IC_{50} values varying between 17 and 35 μM.

Protein kinase C activity was measured in HAP fractions from lung corresponding to the second main peak (equivalent to fractions III and IV from pituitary) and the activity eluting after this (fraction V) and the potency of H7 determined. The Ca^{2+}-independent PKC activity in fraction V was relatively resistant to H7 (IC_{50} value 92 ± 26 μM) while the Ca^{2+}-dependent activity in this fraction was sensitive to H7 (IC_{50} values 9 ± 2 μM)(Figure 4.4). All activity in fraction III/IV (combined) was sensitive to H7 (IC_{50} values of 10 ± 3 μM and 11 ± 5 μM for Ca^{2+}-dependent and Ca^{2+}-independent activity respectively).

Two fractions from αT3-1 cells were also analysed. These corresponded to the two main peaks of PKC activity (fractions 1 and 2 respectively). As shown in Table 4.5, both Ca^{2+}-dependent and Ca^{2+}-independent PDBu-evoked activity in fraction 1 was sensitive to H7 (IC_{50} values 21 ± 8 μM and 21 ± 7 μM respectively). However, fraction 2 contained Ca^{2+}-independent activity that was relatively insensitive to H7 (IC_{50} value 122 ± 32 μM). This activity was, however, highly sensitive to the selective PKC inhibitor Ro 31-8220 (IC_{50} value 78 ± 32 nM)

Cofactor dependence of H7 resistant activity

The cofactor dependence of PKC activity in the fraction from αT3-1 cells containing H7-resistant PKC activity was investigated. Activity was measured in the presence of either PC or PS and the effect of PDBu alone, Ca^{2+} alone and PDBu and Ca^{2+} together were compared with basal activity, as shown in Figure 4.5. Basal activity was similar in the presence of either PC or PS, and there was no increase when PDBu or Ca^{2+} was added

either alone or together, in the presence of PC. When PC was replaced by PS, however, both Ca^{2+} and PDBu alone resulted in increased GS peptide phosphorylation, and there was a further increase when both these cofactors were added together.

Immunoblotting of anterior pituitary hydroxyapatite fractions with isoform-specific antibodies

Pituitary HAP fractions I-V were immunoblotted using antisera for specific PKC isoforms to allow comparison of the distribution of the H7-resistant activity with the elution pattern for each isoform. Figure 4.6 shows immunoblots for PKCs α, β_1, δ, ε, ζ and θ, the isoforms reported to be present in anterior pituitary tissue ((MacEwan, 1993) and D J MacEwan (unpublished data)). Protein kinase C α, β_1, δ, ε and θ were all seen as bands in the 80-94 kDa range. Protein kinase C α immunoreactivity was present in fractions III-V, while fraction I contained the major immunoreactivity for PKCs β_1, δ and ε. PKC ζ immunoreactivity, seen as bands of 81 and 88 kDa, was present in all fractions, the 81 kDa band being strongest in fraction I while the 88 kDa band was predominant in fraction III. The monoclonal anti-PKC θ antibody recognised a 80 kDa band in fraction III similar to that obtained in a Jurkat cell positive control (Baier et al., 1993). This band was also just visible in fraction IV, while in fraction I there was immunoreactivity at approximately 56, 68 and 73 kDa in I, (results not shown) which may represent PKC breakdown products or other cross-reacting proteins. Immunoblots were not performed for either PKC γ or PKC η as no immunoreactivity has been detected for these isoforms in anterior pituitary (Naor et al., 1988)(MacEwan, D. J., Johnson, M. S., Ohno, S. and Mitchell, R; unpublished work).

Autophosphorylation of hydroxyapatite fractions

Hydroxyapatite fractions from both midbrain and pituitary were allowed to autophosphorylate with ATP-γ [^{32}P] in the presence of PS and Ca^{2+}, and the resulting radiolabelled proteins were visualised by autoradiography. In both cases, a number of proteins were labelled in fractions I-IV (Figure 4.7). Prominent bands included, in fraction I, one of 88 kDa and, in fractions III and IV, one of approximately 95 kDa. These may well represent autophosphorylation of PKC β_1 and α respectively. In fraction V from pituitary there was one predominant labelled protein, which had a molecular mass (when phosphorylated) of approximately 140 kDa. This protein was also present in pituitary fraction IV but was not seen in any of the midbrain fractions.

Immunoblotting of hydroxyapatite fractions with PKC consensus antibody

Hydroxyapatite fractions from both pituitary and midbrain were immunoblotted with an antiserum raised to a sequence in the C4 domain that is conserved between all the PKC isoforms (PKC α:[Ac 543-550-Cys]). This was to test whether the phosphorylation signal at 140 kDa represented autophosphorylation of a PKC distinct from the known species recognised by our isoform-specific antisera or whether it might be due to phosphorylation of a PKC substrate that coelutes from HAP with the H7-resistant PKC activity. Although a number of other proteins were labelled by this antiserum, in both anterior pituitary and midbrain HAP fractions, the strongest staining of these was a protein of approximately 130 kDa in anterior pituitary fractions IV and V (Figure 4.8). This immunoreactivity was also present to a lesser degree in fraction III but was not seen in the other pituitary HAP fractions, or in any fractions from

midbrain. This protein may represent the non-autophosphorylated form of the 140 kDa protein which was detected in the autophosphorylation experiments.

The HAP fractions from lung, corresponding to fractions III-V in anterior pituitary, was also immunoblotted with the PKC consensus antibody, as fraction V from this tissue contains H7-resistant PKC activity (Figure 4.4). While a number of proteins were stained in all fractions, there was a distinct band of approximately 130 kDa in fraction V, which was not present in fractions III and IV (Figure 4.9). This band was also present in a fraction eluting immediately after fraction V (data not shown), which contained little other immunoreactivity with this antibody.

Immunoblots to determine the elution pattern of the 130 kDa PKC immunoreactive protein from anterior pituitary

Anterior pituitary extracts were fractionated on a HAP column and samples from each individual fraction (before fractions were pooled) were collected. These were combined in pairs and then immunoblotted with the PKC consensus antibody, to determine the pattern of elution from hydroxyapatite of the 130 kDa protein previously detected in fractions IV and V. As shown in Figure 4.10, immunoreactivity with a molecular mass of 130 kDa first appeared in fraction 32. This corresponded to the beginning of the second main peak of PKC activity (elution volume 105 ml in Figure 4.1b). This immunoreactivity increased throughout the following fractions to reach a peak in fractions 40-42 (corresponding to pooled fraction V) before decreasing to a low level in fraction 56 (elution volume 150 ml in Figure 4.1b). Thus the highest levels of PKC immunoreactivity of approximately 130 kDa corresponded to the pooled

fraction in which H7-resistant PKC activity was most prominent (Table 4.2).

4.4 DISCUSSION

Ca^{2+}-independent PDBu-evoked PKC activity from anterior pituitary tissue was shown in Chapter 3 to contain two components, one of which is relatively resistant to H7, when activity was measured with GS peptide as the substrate. These experiments have shown that this Ca^{2+}-independent activity can be biochemically separated into fractions with different sensitivities to the PKC inhibitor H7. Both preliminary experiments, using DEAE-cellulose chromatography, and more extensive studies using HAP chromatography showed that it was possible to separate the H7-resistant PKC activity from other Ca^{2+}-independent PKC activity (Table 4.1 and 4.2). Hydroxyapatite fractionation of pituitary tissue extracts showed that some PKC activity eluted after the main peak of PKC α (fraction V)(Figure 4.1b), in a position where little PKC activity was detected in midbrain (Figure 4.1a), COS 7 cells (Figure 4.1c) or a number of other tissues (Connor and Clegg, 1993). Hydroxyapatite fractionation of extracts from lung and αT3-1 cells also resulted in some PKC activity eluting in a similar late position to that in pituitary (Figure 4.2). The αT3-1 cell line is derived from anterior pituitary gonadotrophs, the cell type which is responsible for secretion of LH. As LHRH-induced priming of LH secretion and a component of PDBu-evoked LH secretion have been shown to be relatively resistant to H7 (Johnson et al., 1992b, Thomson et al., 1993b), a gonadotroph-derived cell line may represent a good source of the H7-resistant PKC-like kinase. While we were unable to clearly detect H7-resistant PDBu-evoked activity in partially purified cytosolic extracts from these cells (IC_{50} value 38 ± 18; (Johnson et al., 1993)),

evidence from studies on activation of PLD and MAP kinase in αT3-1 cells (Fennell et al., 1994; Sim and Mitchell, 1994) suggested that these cells do contain the H7-resistant PKC. This was supported by preliminary studies on DEAE cellulose fractionation of αT3-1 cell PKC activity (M S Johnson and A J Ison; unpublished data). Lung was also selected for investigation as Ca^{2+}-independent PKC activity in this tissue was shown in Chapter 3 to be relatively resistant to H7. Characterisation of the PKC activity showed that the Ca^{2+}-independent activity in the last-eluting fraction of interest (fraction V) from anterior pituitary and lung (Figures 4.3 and 4.4 respectively) but not midbrain (Table 4.3) was relatively resistant to H7. One previous report has also described PKC activity eluting in this position from rat retinal extracts, although its properties were not assessed in detail (Fujisawa et al., 1992). However we have found no evidence of H7-resistant PKC activity in DEAE cellulose eluates from rat retina (IC_{50} value 18 ± 6 μM)(A J Ison, M. S. Johnson, and R. Mitchell; unpublished data). Ca^{2+}-independent PKC activity in the fourth fraction from pituitary, eluting immediately prior to the H7-resistant fraction, was inhibited by H7 with an intermediate IC_{50} value, probably reflecting the presence of small amounts of the H7-insensitive form of PKC in this fraction. All fractions except fraction II contained Ca^{2+}-dependent activity that was sensitive to H7, which is consistent with a previous report that all the cPKCs are sensitive to this inhibitor (Pelosin et al., 1990). As fraction II contained only immunoreactivity for nPKCs but not cPKCs (Figure 4.6), the absence of Ca^{2+}-dependent activity in this fraction was expected. The Ca^{2+}-dependent activity in fraction V is likely to reflect PKC α, as small amounts of this isoform were detected on immunoblots, although the majority of PKC α was found in fractions III and IV. Of the nPKCs, both PKC δ and ε eluted predominantly in fraction I, while PKC θ eluted mainly

in fraction III. This elution position for PKC δ is consistent with a report of its co-elution with PKC β (Mischak et al., 1991). Immunoreactivity for PKC ζ was generally seen as a doublet of approximately 81 and 88 kDa and was present in all fractions, although only the 88 kDa band was found in fraction V. While the apparent molecular mass of 81 kDa for PKC ζ on SDS-PAGE is consistent with previous reports (Gschwendt et al., 1992; Liyanage et al., 1992), there is some evidence that the 88 kDa band may be due to cross reaction of this antiserum with another PKC isoform (Tsutumi et al., 1993). It is therefore clear that, of PKCs α-θ, only immunoreactivity for PKC α and ζ was present in fraction V, the fraction containing the majority of the H7-resistant activity (Figure 4.6). The Ca^{2+}-dependent activity detected in this fraction is likely to be due to PKC α but the identity of the PKC responsible for the H7-resistant, Ca^{2+}-independent activity is not known. While PKC α may be activated by phorbol esters in the absence of Ca^{2+}, we have shown that PDBu-evoked activity of PKC α purified by HAP chromatography from rat brain, measured in the absence of Ca^{2+}, was inhibited by H7 with normal potency (IC_{50} value 35 ± 16 μM; (Ison et al., 1993)). Since the activity measured in the PKC assay is evoked by PDBu, it is unlikely that the activity of PKC ζ would be detected because phorbol esters are reported to be inactive on this isoform (McGlynn et al., 1992; Nakanishi and Exton, 1992). Furthermore we have evidence that PKC ζ is detected in our assay as basal (not PDBu-evoked) activity and GS peptide, the substrate used in this study, is not a good substrate for this isoform (Johnson et al., 1993). The possibility remains, however, that modified states of PKC α-θ could be present, as modification in the region recognised by the antibody may prevent antigen recognition. Furthermore it is possible that PKCs ι (λ) and μ may be present in this fraction. Protein kinase C ι

shows 72% sequence homology with PKC ζ and it has been reported that this isoform is also recognised by PKC ζ antisera. It is therefore possible that the PKC ζ immunoreactivity detected is, at least in part, due to cross reactivity with PKC ι(λ). Preliminary immunoblots with a PKC μ-specific antiserum showed no evidence of immunoreactivity for this isoform in any of the anterior HAP fractions (R Mitchell and J Pfeilschifter; unpublished data). However these isoforms are unlikely to be responsible for the H7-resistant activity as both PKC ι (λ) and μ are aPKCs (Johannes et al., 1994; Selbie et al., 1993) and are reported to be unable to bind PDBu (Akimoto et al., 1994; Johannes et al., 1994).

In αT3-1 cells Ca^{2+}-independent PDBu-evoked PKC activity in one fraction was also found to be H7-resistant. However this fraction was collected from the second main peak of PKC activity. While this is slightly earlier than H7-resistant activity was detected on elution of pituitary PKCs, there is some evidence to suggest that the precise elution pattern of PKC isoforms may vary depending on the tissue source. For example, PKC ε from rabbit brain was found to elute in earlier fractions than PKC βI/II (Saido et al., 1992), while the main peak of PKC β immunoreactivity from rat retina preceded the main peak of PKC ε immunoreactivity (Fujisawa et al., 1992). Elution of PKC δ from HAP showed immunoreactivity for this isoform from rat brain eluted as a broader peak than recombinant PKC δ expressed in COS 7 cells, even though in both cases the enzyme was detected as a doublet on immunoblots (Ogita, 1992). Further fractionation of αT3-1 cell PKCs into smaller fractions could be conducted to compare the elution profile of the H7-resistant PKC with that of the other PKCs present, for example PKC α. Indeed, immunoblots on unpooled pituitary HAP fractions, using a PKC consensus antibody, showed that the 130 kDa protein thought to

represent the H7-resistant kinase (see later in discussion) was present throughout the second main peak of PKC activity (Figure 4.10). Thus detection of H7-resistant PKC activity in fractions corresponding to this peak is likely to dependent on the quantity of this kinase that is present and the nature of the other PKCs that are also present in the same fraction.

The possibility that the kinase responsible for the H7-resistant activity is not a PKC had to be considered, as both histone IIIS and GS peptide are not PKC-specific substrates, although this seems unlikely as the activity measured is PDBu-evoked. Ca^{2+}-independent activity in the H7-resistant fraction from both pituitary and αT3-1 cells was sensitive to inhibition by the specific PKC inhibitor Ro31-8220 (IC_{50} values 78-130 nM). When the cofactor dependence of this fraction from αT3-1 cells was investigated, there was no PDBu-evoked activity when PC replaced PS. Phosphatidylcholine is unable to act as a cofactor in PKC activation (Nishizuka, 1984b) so from the PS dependence, PDBu activation and Ro 31-8220 inhibition of this kinase activity it seems likely that the H7-resistant kinase is a form of PKC.

In order to detect proteins that may be responsible for the H7-resistant activity, autophosphorylation of HAP fractions was undertaken and the resulting labelled proteins were visualised by autoradiography (Figure 4.7). In fractions I-III, a number of proteins were detected but clear bands were present at molecular masses appropriate for autophosphorylated PKC α and β (95 and 88 kDa respectively). In fraction V from anterior pituitary, there was only one clear band, at approximately 140 kDa. This was also present, though less prominent, in fraction IV but was not seen in the comparable fractions from midbrain. It is therefore possible that this 140 kDa band represents the H7-resistant

kinase, as its distribution between the pituitary HAP fractions correlates with that of the H7-resistant activity. Furthermore, this autophosphorylation signal was also detected in the corresponding HAP fractions from lung (McCulloch et al., 1994), which also contain H7-resistant PKC activity (Figure 4.4). Immunoblotting of midbrain, lung and pituitary fractions III-IV with a consensus antibody designed to recognise all PKC isoforms showed strong staining of an approximately 130 kDa protein in fractions IV and V from pituitary and fraction V from lung. Other pituitary and lung fractions and all midbrain fractions failed to show this band. This is consistent with the idea that the 140 kDa phosphorylated protein may represent the H7-resistant PKC itself rather than a coeluting substrate. Furthermore, detailed immunoblot analysis to investigate the HAP elution profile of the 130 kDa band showed that, while it was present in small amounts throughout the second main peak of PKC activity, it increased to reach maximum intensity at a position corresponding to fraction V, (where H7-resistant activity was most prominent), and decreased in subsequent fractions (Figure 4.10). While this molecular mass of 130 kDa is somewhat larger than that of the well characterised PKC isoforms (PKC α-θ) as well as for PKC ι (λ)(Selbie et al., 1993), reports are now emerging of PKCs that are larger in size. A novel, membrane-bound isoform, PKC μ has been reported in human placenta and several carcinoma cell lines and this isoform is a 117 kDa protein (Johannes et al., 1994). Furthermore, a 160 kDa form of PKC apparently related to the ζ isoform has been detected in hippocampus by immunoblotting (Sacktor et al., 1993). Some studies have also detected, in both hippocampus and platelets, a form of PKC η, designated η', which has a higher molecular mass than the standard PKC η detected in lung and skin (97 kDa rather than 82 kDa)(Sublette et al., 1993a, Wang et al.,

1993). Experiments have been carried out to clarify whether this 130 kDa protein may be responsible for the H7-resistant activity, by fractionating partially-purified pituitary cytosol using centrifugal molecular mass cut-off filters. This showed that PDBu-evoked Ca^{2+}-independent activity with molecular mass greater than 100 kDa contained an H7-resistant component (McCulloch et al., 1994) that was sensitive to the selective PKC inhibitors Ro 31-8220 and GF109203X (D A McCulloch and R Mitchell, ; unpublished data). Furthermore size fractionation of pituitary PKCs using a gel filtration column detected H7-resistant PKC activity in fractions containing protein of molecular mass 120-150 kDa.

In summary, the H7-resistant kinase detected in anterior pituitary tissue in Chapter 3, can be partially separated from other pituitary PKCs by HAP chromatography. This elution profile of H7-resistant PKC activity did not correlate with strong immunoreactivity for PKC α-θ, although it was coincident with an autophosphorylation signal of approximately 140 kDa and PKC immunoreactivity of approximately 130 kDa. This 130 kDa PKC immunoreactivity, like the H7 resistant PKC activity, was present in pituitary and lung but not midbrain. Thus the H7-resistant PKC in anterior pituitary can be distinguished from the well characterised PKCs by both size and biochemical properties and may represent a novel PKC isoform.

Table 4.1. Effect of H7 on Ca^{2+}-independent PKC activity in pituitary DEAE-cellulose eluates.

NaCl concentration (mM)	IC_{50} value (μM)
0-50	13 ± 7
50-100	16 ± 3
100-150	77 ± 11
150-200	N.D.

Phosphatidylserine-dependent thiophosphorylation of GS peptide was evoked by 1 μM PDBu at six concentrations of H7 (0.01-300 μM) in the absence (<3 nM) of Ca^{2+} and the IC_{50} value determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D., not determined.

Table 4.2. Effect of H7 on PDBu-evoked PKC activity in hydroxyapatite fractions from anterior pituitary

HAP fractions	IC_{50} value (μM)	
	Ca^{2+}-independent activity	Ca^{2+}-dependent activity
I	9 ± 2	10 ± 1
II	18 ± 6	N.D.
III	15 ± 8	18 ± 6
IV	45 ± 19	23 ± 5
V	85 ± 10	15 ± 8

Phosphatidylserine-dependent phosphorylation of GS peptide (200 μM) was evoked by 1 μM PDBu at six concentrations of H7 (0.01-300 μM) in the presence (100 μM free) or absence (<3 nM) of Ca^{2+} and the IC_{50} value determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D.,not detectable.

Table 4.3 Effect of H7 on PDBu-evoked PKC activity in hydroxyapatite fractions from midbrain

HAP fractions	IC_{50} value (μM)
I	35 ± 5
II	25 ± 7
III	17 ± 4
IV	23 ± 5
V	33 ± 8

Phosphatidylserine-dependent thiophosphorylation of GS peptide (200 μM) was evoked by 1 μM PDBu at five concentrations of H7 (0.03-300 μM) in the absence (<3 nM) of Ca^{2+} and the IC_{50} value determined by non-linear curve fitting (P. Fit) as described in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$).

Table 4.4. Effect of H7 on PDBu-evoked PKC activity in HAP fractions from rat lung

Fraction	IC_{50} value (μM)	
	Ca^{2+}-independent activity	Ca^{2+}-dependent activity
III/IV	11 ± 5	10 ± 3
V	92 ± 26	9 ± 2

Phosphatidylserine-dependent thiophosphorylation of GS peptide (200 μM) was evoked by 1 μM PDBu at six concentrations of H7 (0.01-300 μM) in the presence (100 μM free) or absence (<3 nM) of Ca^{2+} and the IC_{50} value determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$).

Table 4.5. Effect of H7 on PDBu-evoked PKC activity in HAP fractions from αT3-1 cells.

Fraction	IC_{50} value (μM)	
	Ca^{2+}-independent activity	Ca^{2+}-dependent activity
1	21 ± 8	21 ± 7
2	122 ± 32	48 ± 5

Phosphatidylserine-dependent thiophosphorylation of GS peptide (200 μM) was evoked by 1 μM PDBu at various concentrations of H7 in the presence (100 μM free) or absence (<3 nM) of Ca^{2+} and the IC_{50} value determined by non-linear curve fitting (P. Fit) as descibed in Section 2.6. Values are means ± S.E.M. ($4 \leq n \leq 6$).

Figure 4.1 Elution profiles of PKC activity from hydroxyapatite column

Tissue extracts were prepared from (a) rat midbrain, (b) rat anterior pituitary and (c) COS 7 cells and fractionated by HAP chromatography as described in the Section 2.5. Protein kinase C activity was measured as the amount of GS peptide (150 μM) phosphorylation in the presence of PS and Ca^{2+} that could be inhibited by PKC α_{19-31} (pseudosubstrate inhibitor peptide; final concentration 420 μg/ml). Fractions I-V were collected from pituitary and midbrain fractionation as indicated by the bars. Typical values for the highest peak of activity were 18 $x10^3$ dpm per assay from midbrain and 12.5 x 10^3 dpm per assay from anterior pituitary (with approximately 2 fold greater specific activity of label). The profiles are representative of those from at least 3 separate experiments in each case.

Figure 4.1

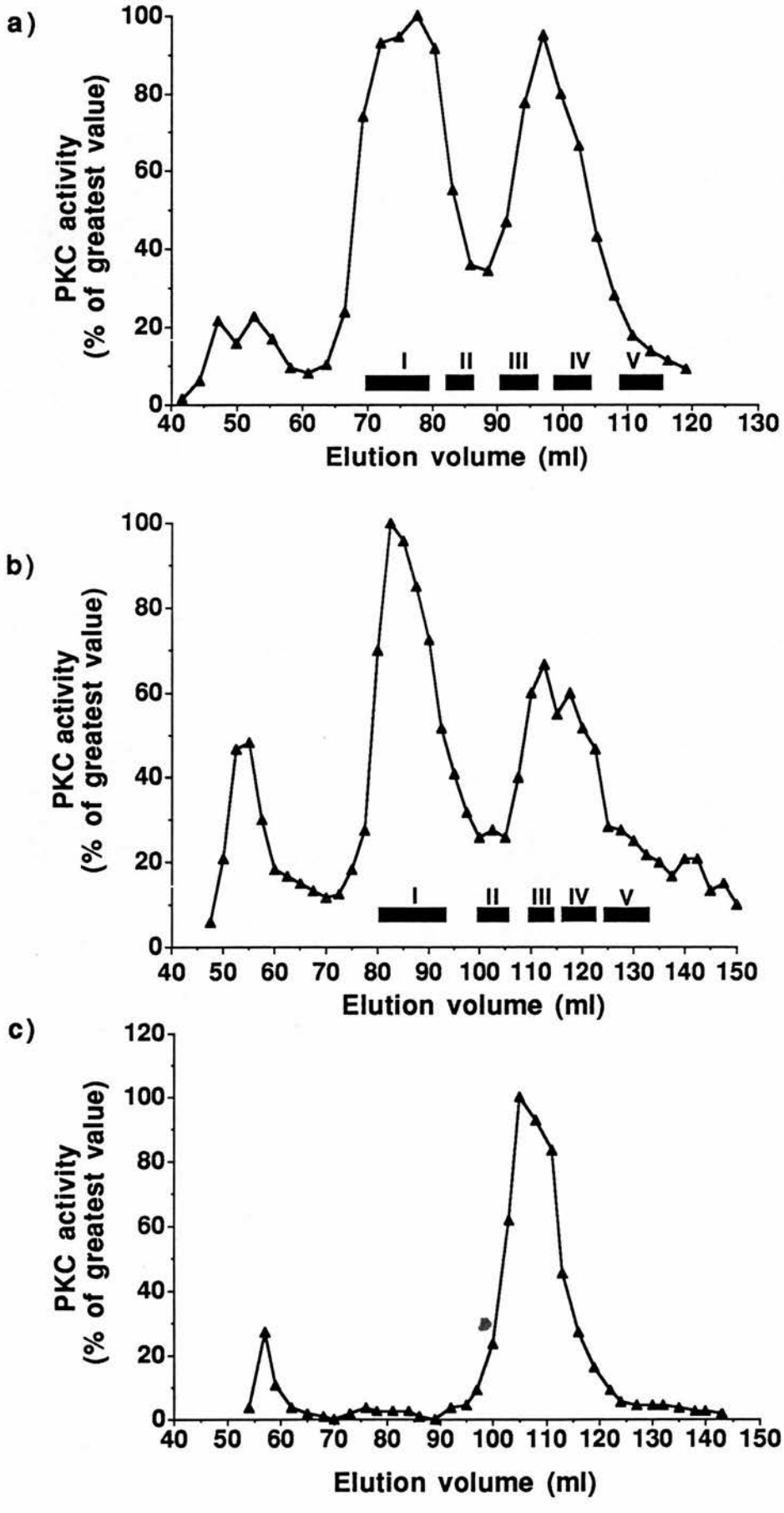
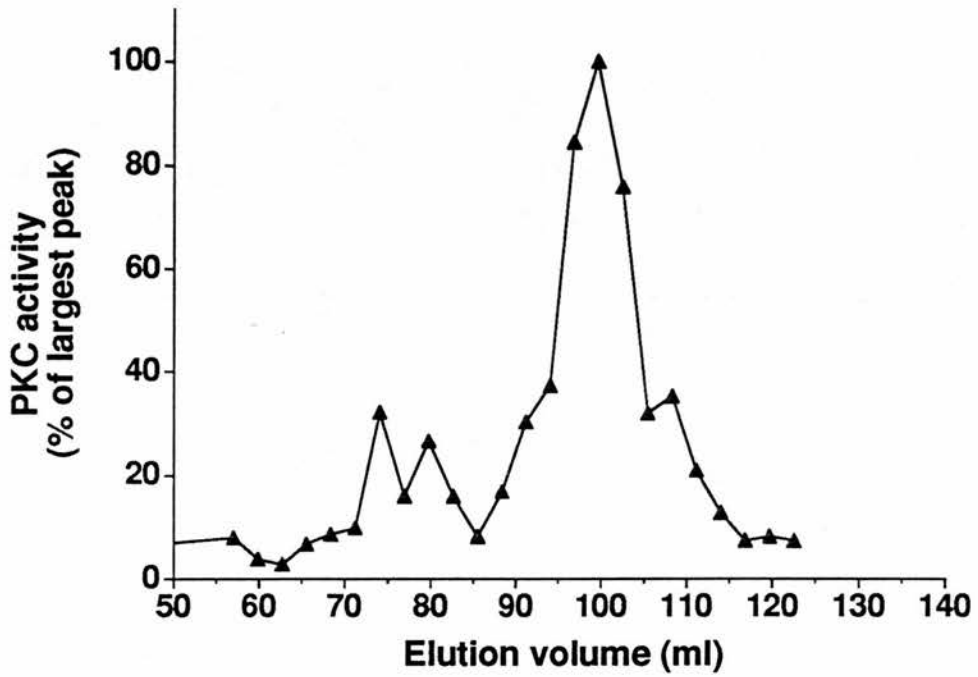

a)
PKC activity
(% of greatest value)
100
80
60
40
20
0
I
II
III
IV
V
40 50 60 70 80 90 100 110 120 130
Elution volume (ml)
b)
PKC activity
(% of greatest value)
100
80
60
40
20
0
I
II
III
IV
V
40 50 60 70 80 90 100 110 120 130 140 150
Elution volume (ml)
c)
PKC activity
(% of greatest value)
120
100
80
60
40
20
0
40 50 60 70 80 90 100 110 120 130 140 150
Elution volume (ml)

Figure 4.2 Elution profiles of PKC activity from hydroxyapatite column

Tissue extracts were prepared from (a) the αT3-1 gonadotroph cell line and (b) rat lung and fractionated by HAP chromatography as described in the Section 2.5. Protein kinase C activity was measured as the amount of phosphorylation of GS peptide (150 μM) in the presence of PS and Ca^{2+} that could be inhibited by PKC α_{19-31} (pseudosubstrate inhibitor peptide; final concentration 420 μg/ml). Typical values for the highest peak of activity were 6.1 x 10^3 dpm per assay from αT3 cells and 20 x 10^3 dpm per assay from lung (both with approximately 2 fold greater specific activity of label than midbrain (Figure 4.1)). The profiles are representative of those from at least 3 separate experiments in each case.

Figure 4.2

a)


PKC activity
(% of largest peak)
0
20
40
60
80
100
50
60
70
80
90
100
110
120
130
140
Elution volume (ml)

b)

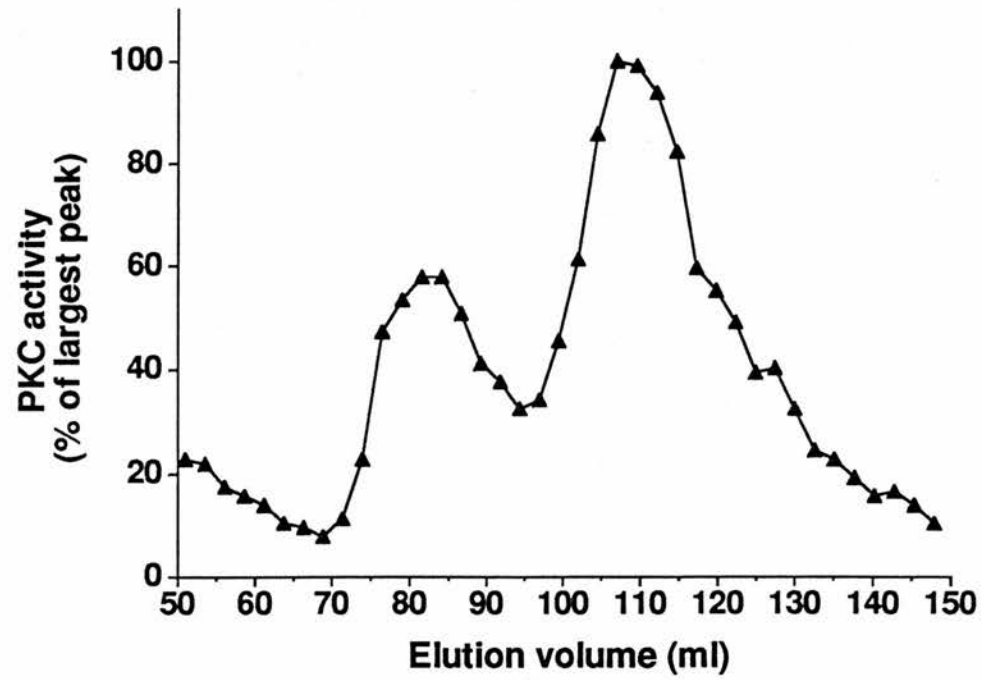

PKC activity
(% of largest peak)
0
20
40
60
80
100
50
60
70
80
90
100
110
120
130
140
150
Elution volume (ml)

Figure 4.3 Inhibition by H7 of PKC activity from anterior pituitary hydroxyapatite fraction V.

Phorbol 12, 13-dibutyrate (1 μM)-evoked PKC activity was measured at various concentrations of H7 both in the presence and absence of Ca^{2+} (100 μM and <3 nM free Ca^{2+} respectively). All points are means ± S.E.M. ($4 \leq n \leq 6$). Ca^{2+}-independent activity was subtracted from the activity in the presence of Ca^{2+} to obtain Ca^{2+}-dependent activity,as described in Section 2.6. (●) Ca^{2+}-independent, (■) Ca^{2+}-dependent activity

Figure 4.3

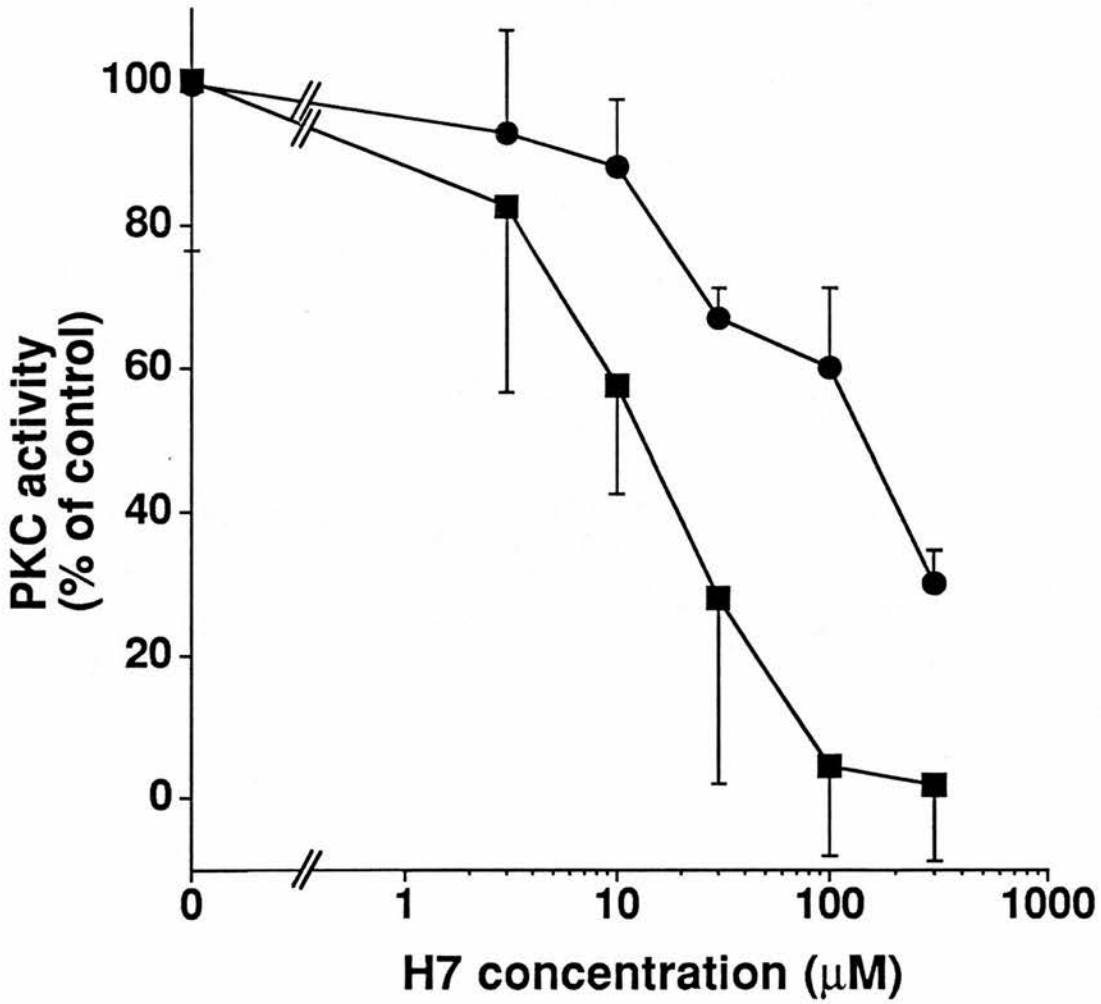

PKC activity
(% of control)
100
80
60
40
20
0
0
1
10
100
1000
H7 concentration (μM)

Figure 4.4 Inhibition by H7 of PKC activity from lung eluting from hydroxyapatite in a position equivalent to pituitary fraction V.

Phorbol 12, 13-dibutyrate (1 μM)-evoked PKC activity was measured at various concentrations of H7, both in the presence and absence of Ca^{2+} (100 μM and <3 nM free Ca^{2+} respectively). All points are means ± S.E.M. ($4 \leq n \leq 6$). Ca^{2+}-independent activity was subtracted from the activity in the presence of Ca^{2+} to obtain Ca^{2+}-dependent activity as described in Section 2.6 . (●) Ca^{2+}-independent, (■) Ca^{2+}-dependent activity

Figure 4.4

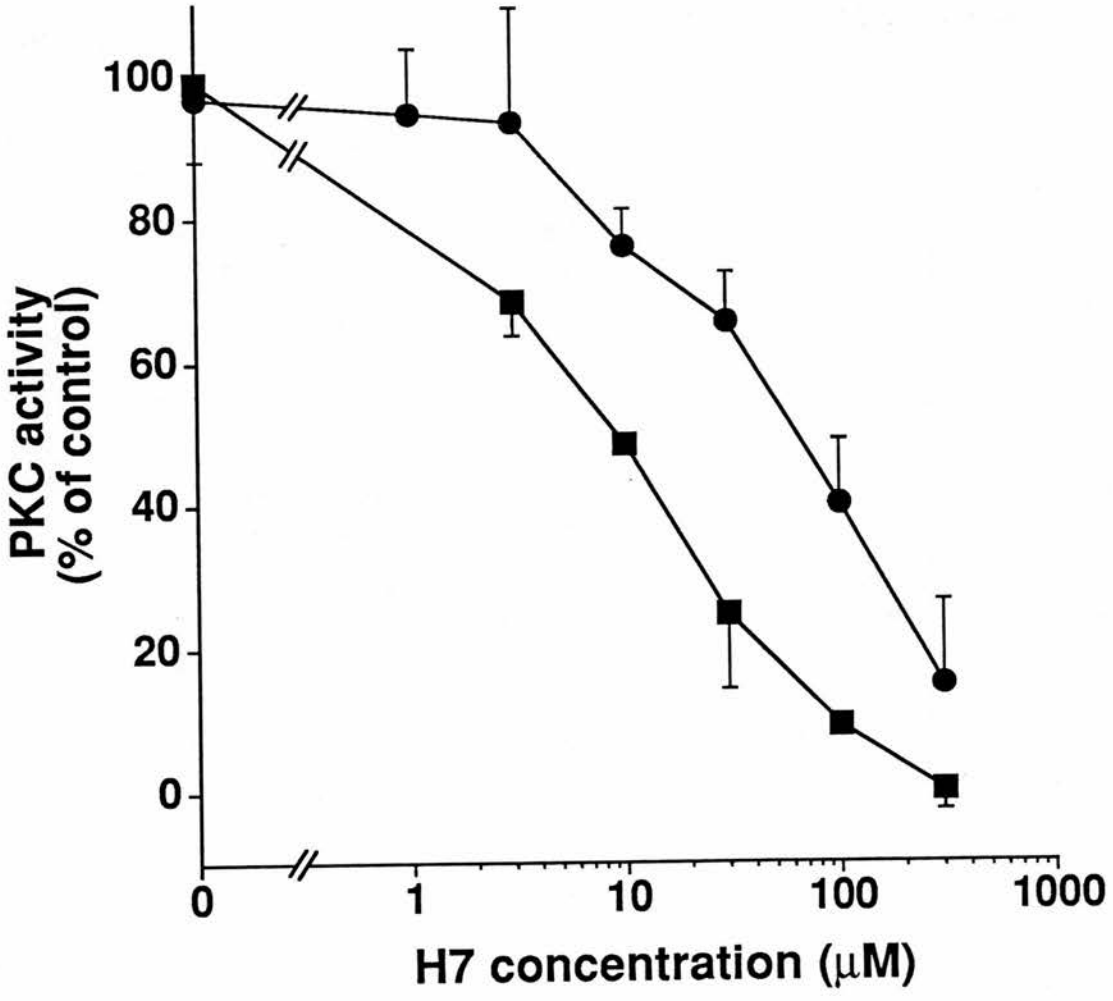

100
80
60
40
20
0
PKC activity
(% of control)
0
1
10
100
1000
H7 concentration (μM)

Figure 4.5 Cofactor dependence of H7-resistant PKC activity

PKC activity in the HAP fraction from αT3-1 cells which contained the H7-resistant activity (see Table 4.5) was measured in the presence of either phosphatidylcholine (PC) or phosphatidylserine (PS)(200 μg/ml). The effect of PDBu (1 μM) and Ca^{2+} (100 μM free Ca^{2+}) either alone or in combination were determined under both conditions. All points are means ± S.E.M. (n=3)

Figure 4.5

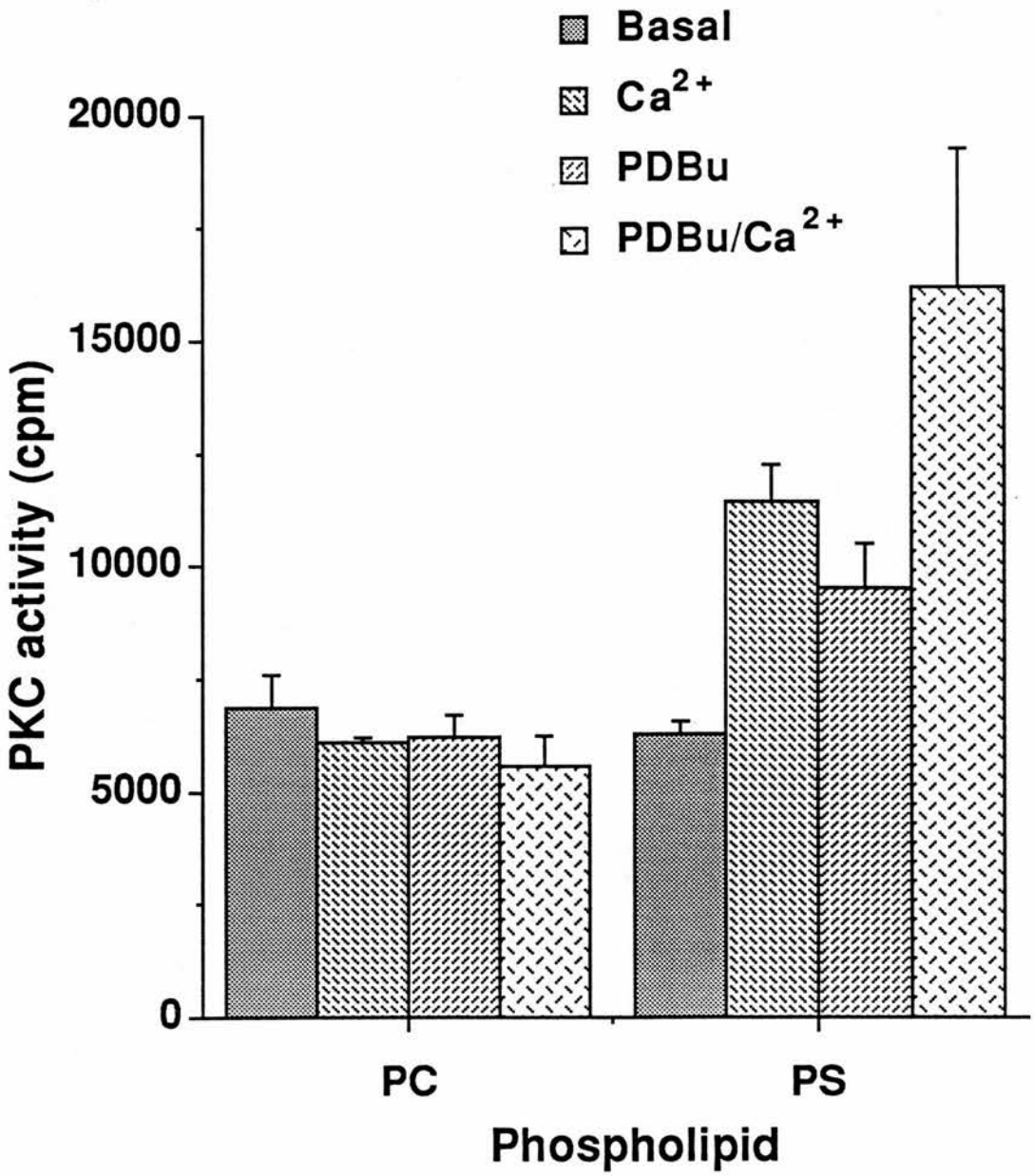

Basal
Ca2+
PDBu
PDBu/Ca2+
20000
15000
10000
5000
0
PKC activity (cpm)
PC
PS
Phospholipid

Figure 4.6 Immunoblots of anterior pituitary hydroxyapatite fractions for PKC isoforms

Anterior pituitary HAP fractions were immunoblotted with antisera specific for PKC α, β_1, δ, ε, θ and ζ as described in the Section 4.2. The relative amounts of protein that were loaded onto SDS-PAGE gels for immunoblotting (the product of sample volume loaded and sample dilution/concentration) were 40:40:1:4:40:40 for anti-PKC α:β1:δ:ε:ζ:θ.

Figure 4.6

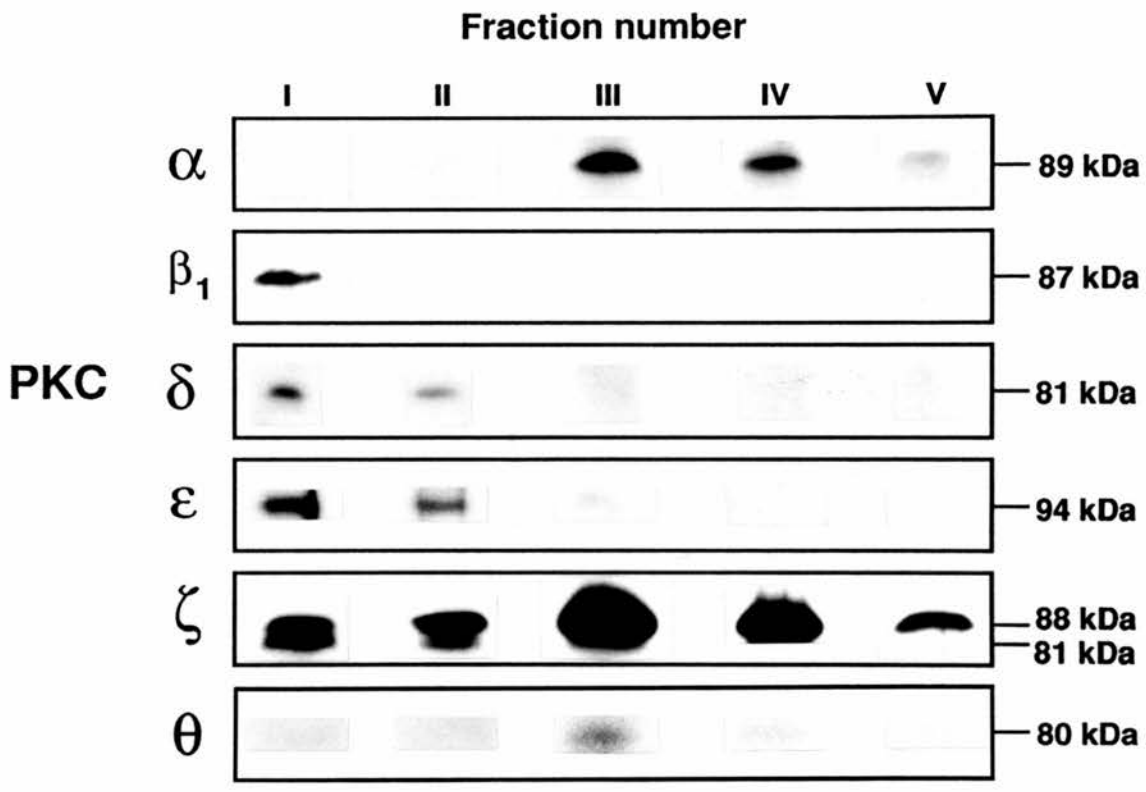

Fraction number
I
II
III
IV
V
PKC
α
β1
δ
ε
ζ
θ
89 kDa
87 kDa
81 kDa
94 kDa
88 kDa
81 kDa
80 kDa

Figure 4.7 Autophosphorylation autoradiographs of hydroxyapatite fractions III-V from midbrain and anterior pituitary

Autophosphorylation of HAP fractions III-V from midbrain and anterior pituitary was carried out in the presence of PS and Ca^{2+} (5.6 μM), the labelled products being visualised by autoradiography. Phorbol esters were not required to activate PKC under these conditions as there was no detergent present (Hannun and Bell, 1986). In pituitary but not midbrain, a strong band was visible at approximately 140 kDa, mainly in fraction V (large arrow). In pituitary fractions III and V and midbrain fraction III, the presumed autophosphorylation of PKC α was visible at about 95 kDa (small arrow).

Figure 4.7

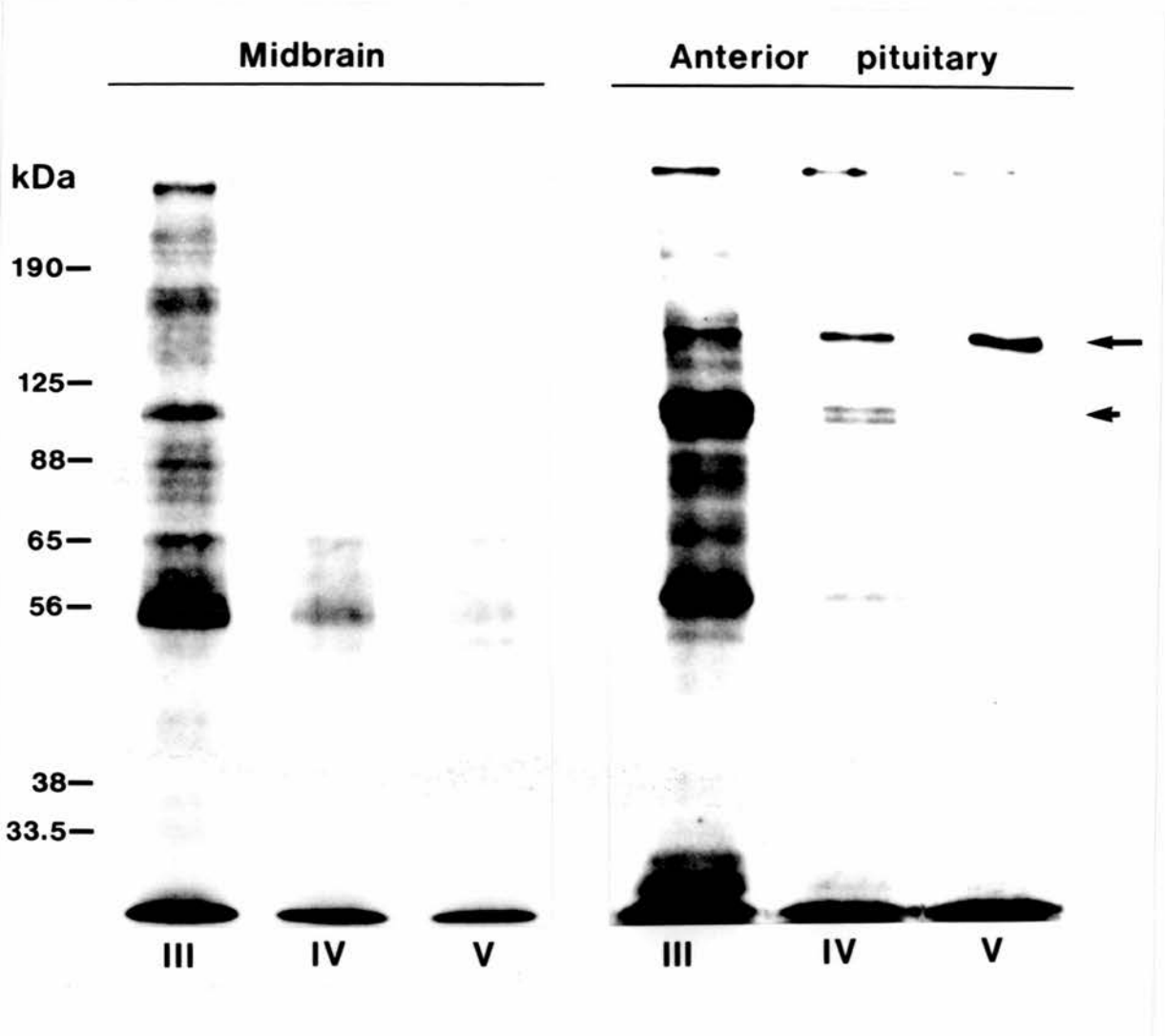

Midbrain
Anterior pituitary
kDa
190—
125—
88—
65—
56—
38—
33.5—
III
IV
V
III
IV
V

Figure 4.8 Immunoblots of hydroxyapatite fractions from anterior pituitary and midbrain with a PKC consensus antibody.

Hydroxyapatite fractions I-V from pituitary and midbrain were immunoblotted with a polyclonal antiserum raised to a PKC consensus sequence in the catalytic domain, PKC α:[Ac 543-550-Cys]. Fractions IV and V from pituitary contained an immunoreactive protein of 130 kDa that was not seen in midbrain or the other pituitary fractions (large arrow). A number of other bands were also apparent in both midbrain and pituitary, including several in the region of 90 kDa (small arrow).

Figure 4.8

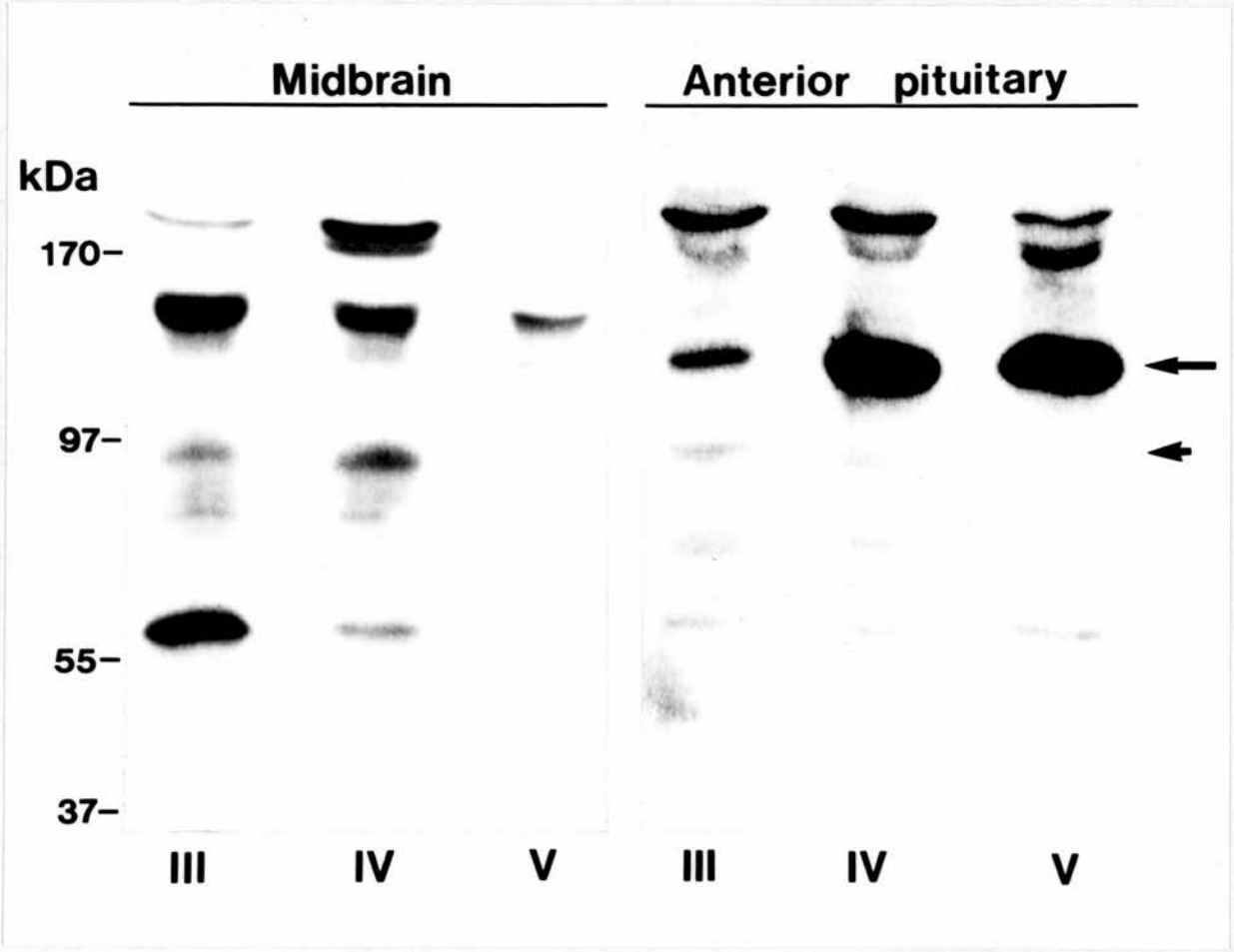


Figure 4.9 Immunoblots of hydroxyapatite fractions from lung with a PKC consensus antibody.

Hydroxyapatite fractions I-V from lung were immunoblotted with a polyclonal antiserum raised to a PKC consensus sequence in the catalytic domain, PKC α:$_{[Ac\ 543\text{-}550\text{-}Cys]}$. Fraction V from lung contained an immunoreactive protein of 130 kDa that was not seen fractions I-IV (large arrow). A number of other bands were also apparent in both midbrain and pituitary, including several in the region of 90 kDa.

Figure 4.9

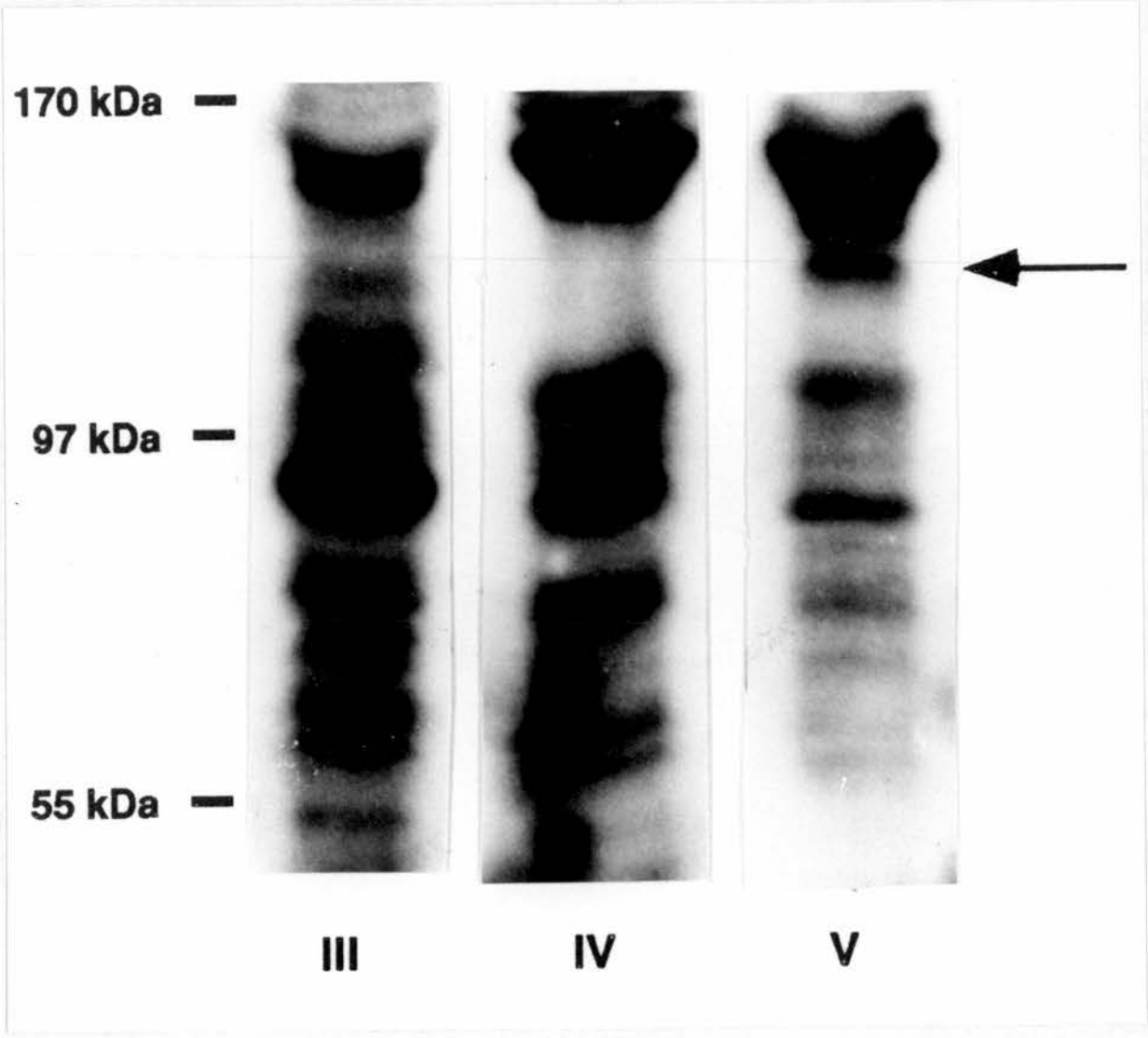
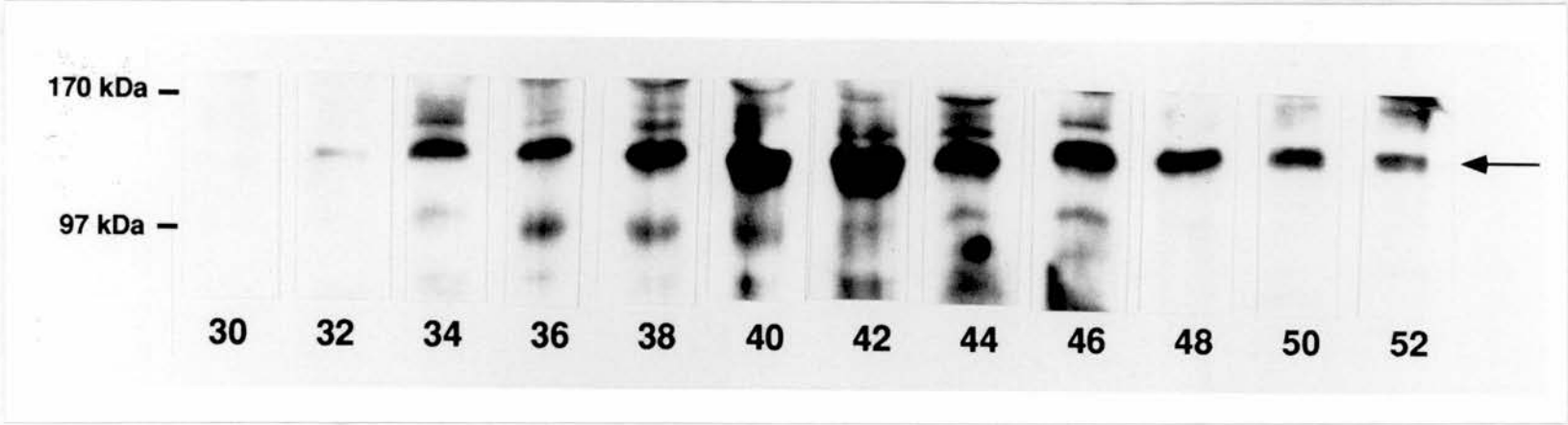


Figure 4.10 Immunoblots of unpooled hydroxyapatite fractions from anterior pituitary with a PKC consensus antibody.

Hydroxyapatite fractions from anterior pituitary were collected throughout elution with a 5-300 mM potassium phosphate gradient. 0.5 ml of every second fraction was combined with 0.5 ml of the subsequent fraction and then concentrated as described in Section 2.7 before immunoblotting with a polyclonal antiserum raised to a PKC consensus sequence in the catalytic domain, PKC α:$_{[Ac\ 543\text{-}550\text{-}Cys]}$. An immunoreactive protein of 130 kDa first appeared at fraction 32, reaching a peak at fraction 40-42 and decreasing by fraction 52 (large arrow). A number of other bands were also apparent in both midbrain and pituitary, including several in the region of 90 kDa.

Figure 4.10



CHAPTER 5

INVESTIGATION OF PKC ζ-RELATED PKCS IN ANTERIOR PITUITARY

5.1 INTRODUCTION

Although PKC ζ was first identified in 1988; (Ono et al., 1988), it is only more recently that it has been classified separately from the other Ca^{2+}-independent PKC isoforms, as a member of the atypical group of PKCs (Nishizuka, 1992). Protein kinase C ζ was the first PKC isoform to be identified that had only one cysteine-rich region in the C1 domain (Ono et al., 1988), and there has been much research conducted to determine how this isoform is regulated (Zhou et al., 1994). Initial transfection experiments, using COS cells, found that PKC ζ was unable to bind phorbol esters (Ono et al., 1989b) and this was supported by evidence from recombinant PKC ζ, produced using a baculovirus expression system (Goodnight et al., 1992), which was also found to be active in the absence of phospholipid (Liyanage et al., 1992; McGlynn et al., 1992). When native PKC ζ was purified from both bovine kidney (Nakanishi and Exton, 1992) and mouse epidermis (Gschwendt et al., 1992), phorbol esters and DAG were once again found to have no effect on the activity of this isoform, although, in both these cases, it was stimulated by phospholipid. Thus it appears that *in vitro* this isoform shows no dependence on phorbol esters for activation.

Phorbol esters and DAG bind to the C1 domain of PKC in a phospholipid-dependent manner (Burns and Bell, 1991). This domain contains cysteine-rich sequences which resemble the zinc finger motif found in DNA binding proteins but, in PKC, they are thought to comprise the phorbol binding site (Burns and Bell, 1991). In all the cPKCs (α-γ) and nPKCs (δ-η), there is a tandem repeat of two cysteine-rich regions while in PKC ζ only the first cysteine motif is present. Deletion mutants of PKC γ have shown that either the first or second cysteine region alone is

capable of binding phorbol esters, although binding was attenuated 10-20 fold when compared to native PKC γ (Burns and Bell, 1991). On the basis of sequence alignment of PKCs and the other PDBu-receptors, a consensus sequence of 15 amino acids required for phorbol binding has been proposed (Ahmed et al., 1991). Analysis of the C1 domain of PKC ζ shows that this region varies by only one amino acid residue (glycine instead of proline) from the consensus sequence. Protein kinase C δ and θ also differ from this sequence by one amino acid, although this is a conservative substitution (isoleucine instead of valine), unlike in PKC ζ, and these isoforms are capable of binding phorbol esters with high affinity. Site-directed mutagenesis of PKC ζ to restore the proline residue and so generate the full consensus sequence did not, however, result in phorbol binding, suggesting that other sequences may be required in addition to this minimal consensus sequence to generate the appropriate conformation for ligand binding (Kazanietz et al., 1994). Another atypical PKC isoform, PKC μ, has recently been cloned that has two cysteine-rich regions which are separated by a 79 amino acid intervening sequence. Both these regions differ from the consensus sequence by one amino acid, and this isoform is reported to be unable to bind phorbol esters (Johannes et al., 1994). Thus the precise requirement for PDBu binding are not yet clearly defined.

While PKC ζ is unable to bind phorbol esters *in vitro* (Nakanishi and Exton, 1992; Ono et al., 1989b), there is controversy surrounding the effects of these compound *in vivo*. Activation of isoforms *in vivo* is often assessed by measuring translocation and down-regulation in response to a stimulus. Several studies have found no evidence of PKC ζ translocation or down-regulation in response to phorbol esters in a variety of different cell types (Gschwendt et al., 1992; Huwiler et al., 1992;

Ways et al., 1992). However, PKC ζ has been found to translocate from the cytosol to the membrane in response to phorbol esters in a variety of cell types including R6 fibroblast cells (Borner et al., 1992b), human platelets (Crabos et al., 1992), neutrophils (Dang et al., 1994) and smooth muscle cells (Mochly-Rosen et al., 1991a). These reports must be treated with caution as there is evidence that PKC ζ-specific antibodies cross-react with cPKCs, either PKC α (Tsutumi et al., 1993; Zhou et al., 1994) or another cPKC distinct from PKC α or β (Batlle et al., 1994). However it would appear that cross-reactivity alone is unable to totally explain all these reports of PKC ζ translocation. In R6 fibroblasts, the presence of PKC ζ was confirmed by both Northern blot analysis and immunoblotting (Borner et al., 1992b). Protein kinase C ζ immunoreactivity was detected at approximately 72 kDa and weak cross reactivity with PKC α was detected at 81 kDa. In response to TPA, the 72 kDa PKC ζ immunoreactivity, in addition to the 81 kDa PKC α, underwent rapid translocation from the cytosol to the membrane fraction. Furthermore, chronic TPA stimulation resulted in total down-regulation of the 72 kDa PKC ζ immunoreactivity. In another study, TPA-stimulated translocation of PKC ζ was detected in human platelets, using an antibody that showed no cross-reactivity with recombinant PKC α (Crabos et al., 1992). Thus response *in vivo* of PKC ζ to phorbol esters cannot be totally dismissed.

Factors other than the ability of PKC ζ to bind phorbol esters may also influence response of this isoform to these compounds *in vivo*. Overexpression of PKC βI has been shown in rat fibroblasts to influence the down-regulation of PKC δ and ε, but not α or ζ, in response to phorbol esters (Borner et al., 1992b), so it is possible that other endogenous isoforms may also effect responses in a particular cell type. In addition it has been reported that expression of either *ras* or *src* may interfere with

the ability of phorbol esters to induce down-regulation of PKC (Lacal et al., 1990; Thompson et al., 1988). The activity of other kinases *in vivo* may also affect the ability of PKCs to respond to phorbol esters, as it has been reported that PKC α requires phosphorylation by an as yet unidentified kinase before it may be activated in a cofactor-dependent manner (Pears et al., 1992). There is also evidence that PKC δ activity may be modulated by tyrosine phosphorylation, although the effect of phosphorylation may vary with different tyrosine kinases, as src was found to stimulate PKC δ more efficiently than fyn (Gschwendt et al., 1994). Thus a variety of factors may affect the ability of PKC *in vivo* to respond to phorbol esters.

There has also been controversy surrounding the molecular mass of PKC ζ. Predictions from the open reading frame of 592 amino acids suggest a molecular mass of 67 kDa but expression of rat PKC ζ in COS 7 cells (Ono et al., 1989b) or in insect cells (McGlynn et al., 1992) resulted in expression of a protein with an apparent molecular mass of 64 and 76 kDa respectively. The major *in vitro* translation product also has an apparent molecular mass of 76 kDa and immunoreactivity of 72-82 kDa has been detected in with anti-PKC ζ antibodies in a variety of cell types (Crabos et al., 1991; Wetsel et al., 1992). The recently cloned human atypical PKC ι shares 72 % identity with human PKC ζ (Selbie et al., 1993) and this isoform cross reacts with anti-PKC ζ antisera (Zhou et al., 1994). This cross reaction may in part be responsible for a number of reports which detect PKC ζ immunoreactivity as a doublet as PKC ι has an apparent molecular mass of 65 kDa (Selbie et al., 1993). Alternatively, PA, unlike other phospholipids, has been shown not only to activate PKC ζ but also to strongly to bind to this PKC (Limatola et al., 1994). This interaction was sufficiently strong to withstand SDS treatment

and result in a electrophoretic mobility shift, from 78 kDa to 80-82 kDa. Thus the presence or absence of PA may be another factor affecting the apparent molecular mass of PKC ζ from a variety of cell types.

Some of the discrepancies concerning the properties of PKC ζ may be a result of the existence of multiple forms of this isoform. It was originally reported that two major RNA transcripts of 2.2 and 4.2 kilobases were present in some rat brain, kidney and lung (Ono et al., 1988), which suggested that this isoform may be alternatively spliced. A recent report has confirmed this and the novel portion has been cloned and sequenced (Powell et al., 1994). In studies of LTP in the hippocampus, immunoblots have also shown the presence of three PKC ζ-related proteins, PKM ζ, native PKC ζ and a high molecular mass form (160 kDa)(Sacktor et al., 1993). Both PKM ζ and the 160 kDa form were significantly increased during maintenance of LTP (Sublette et al., 1993b).

In view of the controversy surrounding the effects of phorbol esters on PKC ζ, the possible existence of multiple forms of this isoform and the reported cross-reactivity of PKC ζ antibodies, it was decided to investigate the presence of aPKCs in the anterior pituitary and the αT3-1 gonadotroph cell line, using a PCR-based strategy. In particular, the possibility of extended forms of aPKCs that may be capable of binding phorbol esters was explored, as such kinases may potentially be responsible for the H7-resistant activity discussed in Chapters 3 and 4.

5.2 Specific Methodology

PCR Methods

The pituitary cDNA library was synthesised by Clontech Laboratories Inc. (Palo Alto, CA, USA) in λZapII. RNA was prepared as

described in Section 2.9. PCR was carried out as described in Section 2.10 using oligonucleotide primers designed as described in Section 5.3. Conditions for PCR amplification using the primers Z1 and Z2 were as follows: Step 1, denaturation at 94°C for 1 min; Step 2, annealing at 50°C for 1 min. This cycle was repeated 35 times, followed by a final cycle; Step 1, denaturation at 94°C for 1 min; Step 2, annealing/extension at 50°C for 8 min, to ensure complete extension of all products. Using primers Z3 and Z4 PCR conditions were as follows: Step 1, denaturation at 94°C for 1 min; Step 2, annealing at 65°C for 1 min. This cycle was repeated 35 times followed by a final cycle; Step 1, denaturation at 94°C for 1 min; Step 2, annealing/extension at 65°C for 8 min. Using primers I1 and I2, initial PCR conditions were as follows: denaturation at 94°C for 1 min and annealing/extension at 60°C for 1 min for 5 cycles; denaturation at 94°C for 1 min and annealing at 63°C for 1 min for 30 cycles and final cycle with denaturation at 94°C for 1 min and annealing at 63°C for 8 min. During subsequent experiments using the same pair of primers, the annealing/extension temperature was decreased to 55°C or 45°C for the first 5 cycles and 60°C or 55°C for the other 31 cycles. One tenth of the amplified PCR products were analysed by agarose gel electrophoresis (Section 2.18.2).

Asymmetric PCR for sequencing PCR products

To enable direct sequencing of a PCR product, asymmetric PCR was conducted to generate a single-stranded product . This method involves adding one primer to the PCR mixture in vast excess over the other, resulting in the generation of an excess of one amplified strand relative to the other. The original PCR product was first purified, using the Wizard method of DNA purification (Section 2.11.1). The reaction mixture

was then prepared as previously described for PCR from cDNA libraries (Section 2.10.2), but one of the primers (Z3) was first diluted either 25 or 50 fold. PCR amplification was then conducted for 30 cycles, using the same conditions as for the original amplification. Products were extracted by adding 0.3 ml chloroform to remove the mineral oil and the upper aqueous layer was removed and retained. The single-stranded product was then selectively precipitated with 4M ammonium acetate (100 µl) and isopropanol (200 µl). After vortexing and incubating at room temperature (10 min), tubes were centrifuged (room temperature, 10 min), and the pellets were washed with 70 % ethanol (500 µl) and dried under vacuum. The pellets were resuspended in distilled water and an aliquot analysed by agarose gel electrophoresis (Section 2.18.2), before the remainder was used as a template for dideoxy sequencing (Section 2.17), using the limiting PCR primer (Z3) as a sequencing primer.

5.3 RESULTS

PCR amplification of PKC ζ-related fragments from anterior pituitary

In order to investigate the possibility of a modified form of PKC ζ, containing an extra sequence in the C1 domain, which may be activated by phorbol esters, a PCR approach was employed. Initially two oligonucleotide primers were designed to regions flanking the C1 domain which are not conserved between PKC ζ and the cPKCs or nPKCs. Potential primer sequences were analysed for complementary nucleotide sequences that might allow dimerisation of the primers or self annealing, the occurrence of which would decrease the efficiency of amplification of PKC-related products. The chosen primers sequences, Z1 and Z2 (shown in Table 5.1) correspond to the amino acid sequences SIYRRGAR (residues 113-120)

and VQTEKHVFE (residues 297-305) respectively. The sites to which these primers bind are located in the pseudosubstrate (Z1) and catalytic domains (Z2)(Figure 5.1), thus the fragment to be amplified would contain a large proportion of the regulatory domain. PCR amplification from both the rat pituitary cDNA library and RNA from the gonadotroph αT3-1 cell line resulted in no products larger than the 577 base pair (bp) fragment predicted from the sequence published for PKC ζ (Goodnight et al., 1992), as shown in Figure 5.2.

PCR amplification using PKC ζ- and PKC ι-specific primers

Subsequent publication of the sequence of another aPKC, PKC ι (Selbie et al., 1993), which is closely related to PKC ζ, revealed that the primers used to detect PKC ζ would also amplify PKC ι, if present, as shown in Figure 5.1, and the resulting product would be of a similar size for both isoforms. In view of this and the cross reactivity of PKC ι with anti-PKC ζ antisera, it was decided to design specific primers to allow the detection of these isoforms separately. The PKC ζ-specific oligonucleotide sequences, Z3 and Z4 (shown in Table 5.1) correspond to the amino acid sequences TSVDAMTT (residues 29-36 in PKC ζ) and PVIDGVD (residues 235-242 in PKC ζ) respectively. These sequences are located in V1 region upstream from the pseudosubstrate domain and in the V3 hinge region linking the regulatory and catalytic domains, as these regions show little homology between PKC ζ and ι, as well as with the other PKC isoforms (Figure 5.3). Degenerate oligonucleotide primers were designed for PKC ι as only the human sequence was published at that time. The sequences of these primers, I1 and I2 (shown in Table 5.1) are also located in the V1 and V3 regions (Figure 5.3) and correspond to the amino acid sequences MSHTVAGG (residues 2-8 in PKC ι) and MHSDHAQT (residues 199-206 in

PKC ι) respectively. The sequence of PKC λ, the mouse homologue of PKC ι, has subsequently been published and the relationship between the primers I1 and I2 and this mouse sequence is shown in Figure 5.4.

PCR amplification of RNA from the gonadotroph αT3-1 cell line using the PKC ζ-specific primers Z3 and Z4 produced only one fragment of approximately the expected length (630 bp)(Figure 5.5). In order to confirm that this product corresponded to PKC ζ as previously published (Goodnight et al., 1992), asymmetric PCR was employed to obtain a single stranded fragment. This involved using the original product as a template for PCR using a large excess of one primer (Z4), as described in Section 5.2. The resulting single-stranded product was sequenced using the limiting primer (Z3), and found to be identical to mouse PKC ζ (Goodnight et al., 1992)(as shown in Figure 5.6).

Initial PCR amplification of RNA from anterior pituitary and αT3-1 cells using the PKC ι specific primers I1 and I2 resulted in no detectable products. As it is possible that the initial PCR conditions were unsuitable for this pair of primers, PCR amplification of cDNA libraries from both rat pituitary and dorsal root ganglion (DRG) neurones was conducted at various levels of stringency (annealing temperatures 45-60°C, as described in Section 5.2). PCR at conditions of low stringency resulted in amplification of a large number of products, which are likely to be the result of the primers annealing non-specifically. Under conditions of intermediate stringency, individual products were amplified but none were of the expected length (613 bp). These products were, however, ligated into the pGEM-T vector and transformed into *E coli.* as described in Sections 2.13-2.15, and DNA sequencing analysis conducted. The resulting sequences, however, showed no homology to PKC ι, any of the other PKC isoforms or any sequence in the GenBank™ and EMBL data bases.

As previously mentioned, primers I1 and I2 were designed using the human PKC ι sequence (Selbie et al., 1993), the only published sequence available for this isoform at that time. Although redundancies were incorporated in the primer sequences to accommodate this, it is possible that these primers are unable to amplify the rat PKC ι sequence even if present in anterior pituitary. To investigate this, PCR amplification was conducted on RNA from rat kidney, as this tissue has been shown to contain PKC ι (Selbie et al., 1993). As shown in Figure 5.7, amplification using the PKC ζ primers Z3 and Z4 resulted in a fragment of the expected length (632 bp) but there was no product using the PKC ι primers, I1 and I2. Amplification of rat kidney RNA was also conducted at a range of magnesium concentrations (2-10 mM), as this can affect the ability of the primers to bind to homologous sequences (Newton and Graham, 1994). However, there were still no products of the expected length amplified with these primers.

5.4 DISCUSSION

The presence of PKC ζ and ζ-related PKCs in anterior pituitary was investigated as immunoreactivity for this isoform is detected in HAP fractions that contain H7-resistant activity (Figure 4.6). Protein kinase C ζ is resistant to lipophilic inhibitors, such as α-tocopherol which is known to interfere with the regulatory domain (Kochs et al., 1993b). There are also some reports that staurosporine, which is thought to interact with the catalytic domain, also shows reduced potency for PKC ζ compared to other PKC isoforms (Kochs et al., 1993b, McGlynn et al., 1992), although there is some conflicting evidence (Gschwendt et al., 1992). While we have evidence to suggest that PKC ζ is detected as baseline activity, in the presence of PS but no PDBu, we have also found that this activity is relatively resistant to H7

(Johnson et al., 1993). Therefore it may be possible that if there is an extended variant of PKC ζ, which is activated by phorbol esters, it could also be relatively insensitive to H7 inhibition. A high molecular mass form of PKC ζ has been detected in hippocampus by immunoblotting (Sacktor et al., 1993), and while no such immunoreactivity was detectable in anterior pituitary or αT3-1 cells, this is likely to depend on the antiserum used. Therefore a PCR approach was selected, which also permits identification of any interesting products by DNA sequencing analysis. Oligonucleotide primers were designed from the sequence of PKC ζ (Ono et al., 1989b) to sites flanking the cysteine-rich region of the C1 domain (Figure 5.1), which should allow amplification of PKC ζ but not PKC α-θ. However, while PCR amplification of RNA from both anterior pituitary and αT3-1 cells resulted in a product of 577 bp, the length predicted from the sequence published for PKC ζ, there were no products of a larger size (Figure 5.2). The PCR primers bind to sites located in the pseudosubstrate domain and in the catalytic domain downstream from the ATP binding site, regions which are conserved in the closely related aPKC, PKC ι(λ)(Figure 5.1), and the resulting amplified fragment contains a large portion of the regulatory domain. Taken together this suggests that there may not be an extended PKC ζ-related kinase present in anterior pituitary.

As previously mentioned, this pair of primers are likely to anneal to both PKC ζ and PKC ι(λ), as these isoforms share identical amino acid sequences at these sites (Figure 5.1). While PKC ζ has been detected in anterior pituitary and αT3-1 cells by immunoblotting (Figure 4.6)(Johnson et al., 1993), PKC ζ antibodies are reported to cross react with PKC ι (Zhou et al., 1994). Western blots detected the presence of PKC ζ immunoreactivity in spleen (Wetsel et al., 1992), although Northern blots detected no mRNA for PKC ζ in this tissue (Ono et al., 1988) and later reports suggest that

spleen lacks PKC ζ but abundantly expresses PKC ι (Selbie et al., 1993; Zhou et al., 1994). While Northern blots provided further evidence for the presence of PKC ζ in anterior pituitary (A Ison, E Lutz and R Mitchell; unpublished data), immunoreactivity for this isoform is present in all the HAP fractions tested and it is possible that this may be partly due to the presence of PKC ι. Therefore, in order to detect PKC ζ and ι separately, specific pairs of primers for each isoform were designed. As these isoforms share 72 % amino acid identity, it was difficult to find regions that were sufficiently different to be sure of specificity. The catalytic domain is particularly homologous, so sites were selected in the more divergent V1 and V3 regions of the regulatory domain (Figure 5.3). Amplification of αT3-1 cell RNA using the PKC ζ-specific primers resulted in a single band of the 632 bp predicted from the mouse PKC ζ sequence (Figure 5.5). DNA sequencing analysis confirmed the identity of this product as PKC ζ. As there were no products of a larger size, this supports the initial result suggesting that, in pituitary gonadotrophs, there are no PKC ζ-related kinases which are extended in the C1 region of the regulatory domain. An alternatively spliced form of PKC ζ has recently been identified in a rat prostatic adenocarcinoma cell line, as well as in rat brain, and the novel portion has been cloned and sequenced (Powell et al., 1994). This form of PKC ζ is extended at the 5'end of the original sequence published for this isoform (Ono et al., 1988), so contains an alternative V1 domain to that of the full length sequence published for PKC ζ (Ono et al., 1989b). As the point of convergence is the pseudosubstrate site at the beginning of the C1 domain, this means that the original pair of primers used to detect PKC ζ (Z1 and Z2) will result in amplification of both forms of PKC ζ, if present in anterior pituitary cells, although the two forms would be indistinguishable. However the second pair of primers (Z3 and Z4) will only result in

amplification of one of these forms, as the recently-identified variant has a different sequence in the V1 domain, the region to which Z3 is designed to bind. While differences in the properties of the two forms of PKC ζ have yet to be explored, modification of the V1 domain seems unlikely to affect the phorbol binding properties of the enzyme.

Amplification using the PKC ι-specific primers I1 and I2 did not result in products encoding PKC ι in any of the tissues or cDNA libraries tested. These primers were tested under a range of PCR conditions including, in some cases, annealing temperatures as low as 45°C for the first few cycles. As the melting temperatures (T_m) of the primers I1 and I2 (calculated as described in Section 2.10) are 69°C and 68°C respectively and the recommended starting point for optimisation of conditions is 5°C below the T_m (Newton and Graham, 1994), the failure of these primers to amplify a product of the expected size (632 bp) is unlikely to be due to inappropriate conditions for the PCR cycle. The concentration of Mg^{2+} in the reaction mix was also varied between 2-10 mM. Mg^{2+} forms a complex with dNTPs which is essential for dNTP incorporation and also stimulates the polymerase activity. The concentration of $MgCl_2$ can influence both the specificity and yield of PCR, as insufficient Mg^{2+} yields leads to low yield while excess Mg^{2+} leads to non-specific products (Newton and Graham, 1994). However the optimal concentration can vary with the primer sequences and the template to be amplified. As PCR using I1 and I2 failed to amplify specific products from kidney RNA, a tissue which has been reported to contain PKC ι (Selbie et al., 1993), while amplification from the same RNA preparation using the PKC ζ specific primers Z3 and Z4 resulted in a product expected of the expected size (Figure 5.7), it is possible that these primers are unsuitable for the amplification of rat PKC ι. These primers were designed from the human sequence for PKC ι, although

degeneracies were incorporated to allow for different codon usage. However subsequent publication of the sequence for PKC λ (Akimoto et al., 1994), the mouse homologue of human PKC ι, shows that there are amino acid substitutions within the sequences to which the primers are designed to bind as shown in Figure 5.3. Analysis of the nucleotide sequence of PKC λ in the sites to which the primers I1 and I2 are designed to bind showed that in each case there are three mismatches, none of which are at the 3' end of the primer sequences (Figure 5.4). During PCR amplification of a DNA template, dNTPs are added to the 3' end of the oligonucleotide primer, which is bound to the template sequence. Where mismatches occur in the primer sequence, it is preferable that they are located towards the 5' end, as mismatches near the 3' end will not be tolerated and the mismatched primer will not be extended (Newton and Graham, 1994). As in this case the mismatches are not near the 3' end, these primers should be capable of amplifying PKC λ. It is, however, not known how closely the rat sequence resembles that of either the mouse or human homologues, so it is possible, if there are be variations in rat sequence at the 3' end of these primer binding sites, that these could prevent amplification from occurring. Alternatively, it is possible that the failure of these primers to amplify a sequence encoding PKC ι may be due to the 3' end of the I1 primer forming a secondary structure, as this sequence contains a number of G and C residues, which may be capable of self-annealing. Therefore to determine whether PKC ι is present in anterior pituitary and αT3-1 cells, it may be necessary to design further sets of primers specific for this isoform, based on similarities between the human and mouse sequences, and optimise the conditions using RNA from a tissue, such as kidney, which expresses high levels of PKC ι.

Table 5.1 PKC isoform-specific oligonucleotide primers used in this study.

Primer	Sequence	Nucleotide positions
Z1 (sense)	5'-TCCATCTACCGCCGTGGAGCC AG-3'	484-507
Z2 (antisense)	5'-CGAACACGTGCTTCTCTGTCTG CAC-3'	1036-1060
Z3 (sense)	5'-ACCAGCGTGGATGCCATGAC AA-3'	108-129
Z4 (antisense)	5'-ATCCACCCCATCGATGACAGG-3'	720-740
I1 (sense)	5'-TCICACACIGT(G/C)GCIGG(G/C) GG-3'	4-23
I2 (antisense)	5'-GTCTG(T/G)GC(A/G)TGGTCAGA (A/G)TGCAT-3'	595-617

Details regarding the specific use of each oligonucleotide are given in Chapter 2 and Section 5.2. Redundancies were inserted where the only sequence available was human, to allow for species variation in codon usage. Nucleotide positions are derived from the cDNA sequence of rat PKC ζ, mouse PKC ζ and human PKC ι for primers Z1 and Z2, Z3 and Z4, and I1 and I2 respectively.

Figure 5.1 Comparison of amino acid sequence of PKC isoforms ζ, ι and λ.

The amino acid sequence of PKC ζ, ι and λ from either mouse (ζ and λ) or human (ι) are shown. The C1-4 and V1-3 domains are labelled at the start of each. Individual PKC isoforms are denoted by the appropriate Greek letter on the left, and the amino acid residues are numbered on the right. The positions of the oligonucleotides used as primers for PCR amplification of PKC ζ are indicated by the shaded boxes;  Z1,  Z2. P, Z and A denote the pseudosubstrate, cysteine-rich zinc finger-like region and ATP binding domains respectively.

Figure 5.1


V1
ι MSHTVAGGGSGDHSHQVRVKAYYRGDIMITHFEPSISFEGLCNEVRDMCS 50
λ MWDTVACGGGGDHSHQVRVKAYYRGDIMITHFEPSISFEGLCSEVRDMCS
ζ .MPSRTDPKMDRSGGRVRLKAHYGGDILITSVDAMTTFKDLCEEVRDMCG
ι FDNEQLFTMKWIDEEGDPCTVSSQLELEEAFRLYELNKDSELLIHVFPCV 100
λ FDNEQPFTMKWIDEEGDPCTVSSQLELEEAFRLYELNKDSELLIHVFPCV
ζ LHQQHPLTLKWVDSEGDPCTVSSQMELEEAFRLVCQGRDEVLIIHVFPSI
C1
P
ι PERPGMPCPGEDKSIYRRGARRWRKLYCANGHTFQAKRFNRRAHCAICTD 150
λ PERPGMPCPGEDKSIYRRGARRWRKLYCANGHTFQAKRFNRRAHCAICTD
ζ PEQPGMPCPGEDKSIYRRGARRWRKLYRANGHLFQAKRFNRGAYCGQCSE
Z
V3
ι RIWGLGRQGYKCINCKLLVHKKCHKLVTIECGRH..SLPQEPVMPMDQSS 200
λ RIWGLGRQGYKCINCKLLVHKKCHKLVTIECGRH..SLPPEPMMPMDQ.T
ζ RIWGLSRQGYRCINCKLLVHKRCHVLVPLTCRRHMDSVMPSQEPPVDGKN
ι ....MHSDHAQTVIPYNPSS..HESLDQVGEE.KEAMNTRESGKASSSLG 250
λ ....MHPDHTQTVIPYNPSS..HESLDQVGEE.KEAMNTRESGKASSSLG
ζ DGVDLPSEETDG.IAYISSSRKHDNIKDDSEDLKPVIDGVDGIKISQGLG
C3/C4
A
ι LQDFDLLRVIGRGSYAKVLLVRLKKTDRIYAMKVVKKELVNDDEDIDWVQ 300
λ LQDFDLLRVIGRGSYAKVLLVRLKKTDRIYAMKVVKKELVNDDEDIDWVQ
ζ LQDFDLIRVIGRGSYAKVLLVRLKKNDQIYAMKVVKKELVHDDEDIDWVQ
ι TEKHVFEQASNHPFLVGLHSCFQTESRLFFVIEYVNGGDLMFHMQRQRKL 350
λ TEKHVFEQASNHPFLVGLHSCFQTESRLFFVIEYVNGGDLMFHMQRQRKL
ζ TEKHVFEQASSNPFLVGLHSCFQTTSRLFLVIEYVNGGDLMFHMQRQRKL
ι PEEHARFYSAEISLALNYLHERGIIYRDLKLDNVLLDSEGHIKLTDYGMC 400
λ PEEHARFYSAEISLALNYLHERGIIYRDLKLDNVLLDSEGHIKLTDYGMC
ζ PEEHARFYAAEICIALNFLHERGIIYRDLKLDNVLLDADGHIKLTDYGMC
ι KEGLRPGDTTSTFCGTPNYIAPEILRGEDYGFSVDWWALGVLMFEMMAGR 450
λ KEGLRPGDTTSTFCGTPNYIAPEILRGEDYGFSVDWWALGVLMFEMMAGR
ζ KEGLGPGDTTSTFCGTPNYIAPEILRGEEYGFSVDWWALGVLMFEMMAGR
ι SPFDIVGSSDNPDQNTEDYLFQVILEKQIRIPRSLSVKAASVLKSFLNKD 500
λ SPFDIVGSSDNPDQNTEDYLFQVILEKQIRIPRSLSVKAASVLKSFLNKD
ζ SPFDIIT..DNPDMNTEDYLFQVILEKPIRIPRFLSVKASHVLKGFLNKD
ι PKERLGCHPQTGFADIQGHPFFRNVDWDMMEQKQVVPPFKPNISGEFGLD 550
λ PKERLGCHPQTGFADIQGHPFFRNVDWDMMEQKQVVPPFKPNISGEFGLD
ζ PKERLGCRPQTGFSDIKSHAFFRSIDWDLLEKKQTLPPFQPQITDDYGLD
ι NFDSQFTNEPVQLTPDDDDIVRKIDQSEFEGFEYINPLLMSAEECV 596
λ NFDSQFTNEPVQLTPDDDDIVRKIDQSEFEGFEYINPLLMSAEECV
ζ NFDTQFTSEPVQLTPDDEDVIKRIDQSEFEGFEYINPLLLSAEESV

Figure 5.2 Agarose gel electrophoresis of the products from reverse transcriptase PCR amplification using primers Z1 and Z2.

10 μl of the reaction mixture after PCR amplification with oligonucleotide primers Z1 and Z2 (Lanes 2 and 3) and control primers (Lane 1) was analysed by electrophoresis through a 1% agarose gel containing 0.5 mg/ml ethidium bromide. A strong band was visible at approximately 577 bp (as indicated by the arrow), the predicted size for PKC ζ, following amplification of αT3-1 cell and anterior pituitary RNA (Lanes 2 and 3 respectively), but no larger fragments were detected. Lane 1 shows amplification of αT3-1 cell RNA using control primers which produced the expected 293 bp fragment. Sizes of the molecular weight markers are indicated in base pairs.

Figure 5.2

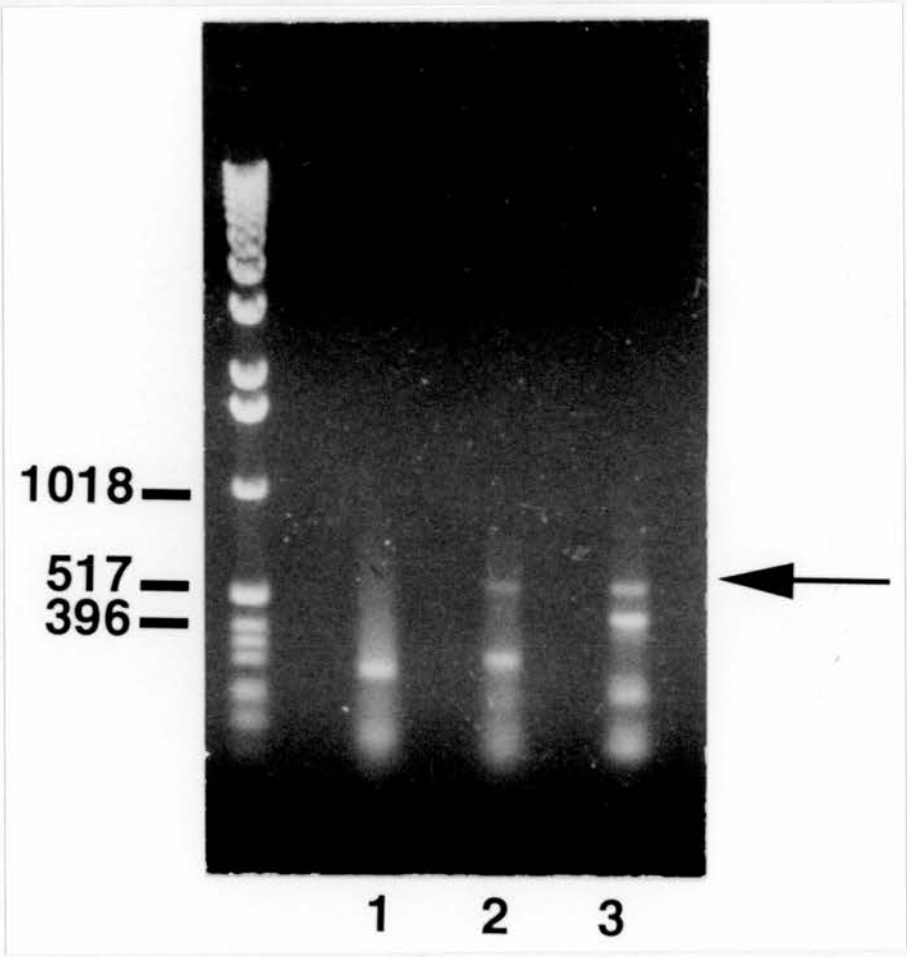


Figure 5.3 Comparison of amino acid sequence of PKC isoforms ζ, ι and λ showing PKC ζ and PKC ι primer locations. The amino acid sequence of PKC ζ, ι and λ from either mouse (ζ and λ) or human (ι) are shown. The C1-4 and V1-3 domains are labelled at the start of each. Individual PKC isoforms are denoted by the appropriate Greek letter on the left, and the amino acid residues are numbered on the right. The positions of the oligonucleotides used as primers for PCR amplification are indicated by the shaded boxes; Z3, Z4, I1, I2. P, Z and A denote the pseudosubstrate, cysteine-rich zinc finger like region and ATP binding domains respectively. Amino acids which vary between PKC ι and λ are indicated by asterisks.

Figure 5.3

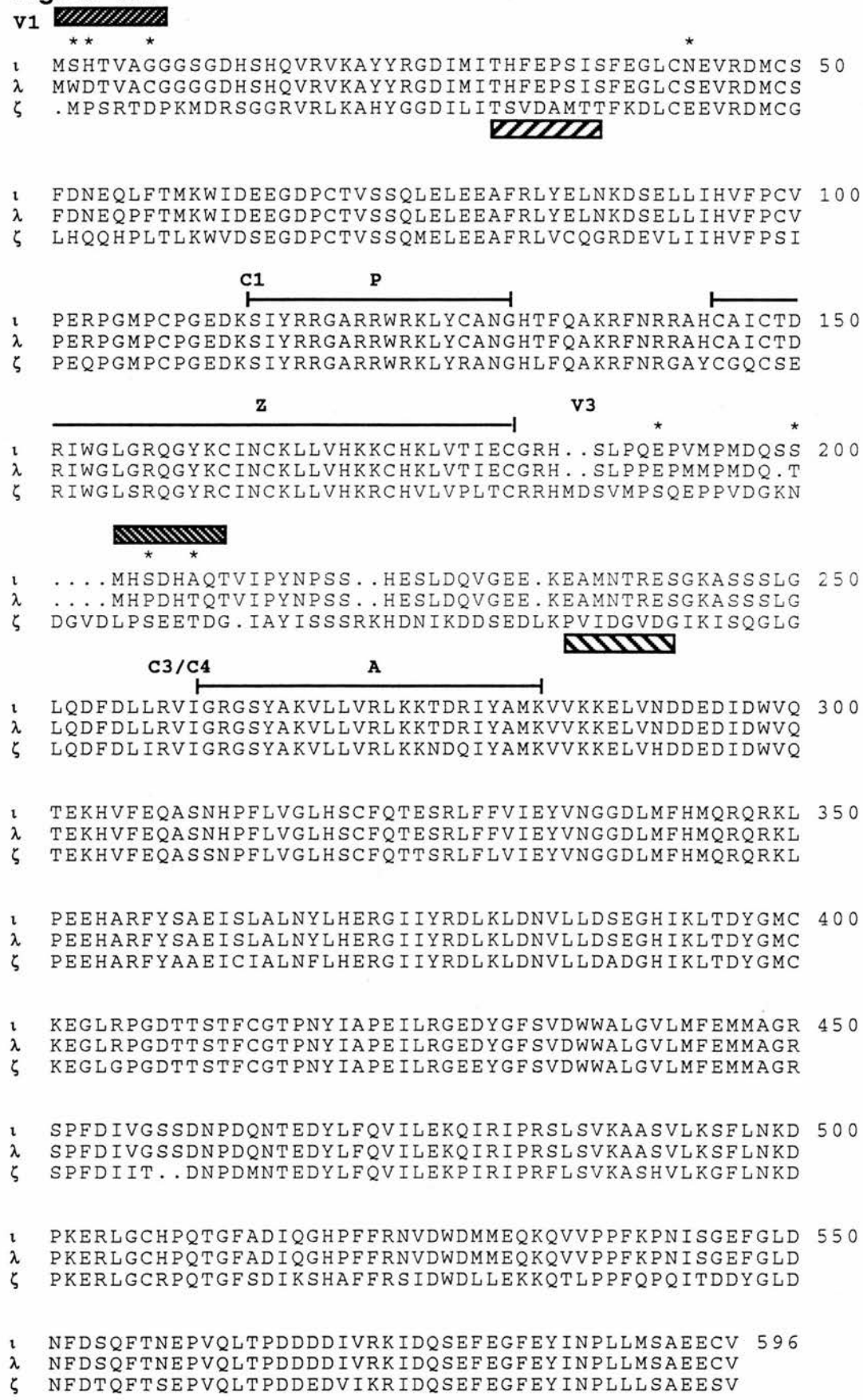

V1
C1
P
Z
V3
C3/C4
A
ι MSHTVAGGGSGDHSHQVRVKAYYRGDIMITHFEPSISFEGLCNEVRDMCS 50
λ MWDTVACGGGGDHSHQVRVKAYYRGDIMITHFEPSISFEGLCSEVRDMCS
ζ .MPSRTDPKMDRSGGRVRLKAHYGGDILITSVDAMTTFKDLCEEVRDMCG
ι FDNEQLFTMKWIDEEGDPCTVSSQLELEEAFRLYELNKDSELLIHVFPCV 100
λ FDNEQPFTMKWIDEEGDPCTVSSQLELEEAFRLYELNKDSELLIHVFPCV
ζ LHQQHPLTLKWVDSEGDPCTVSSQMELEEAFRLVCQGRDEVLIIHVFPSI
ι PERPGMPCPGEDKSIYRRGARRWRKLYCANGHTFQAKRFNRRAHCAICTD 150
λ PERPGMPCPGEDKSIYRRGARRWRKLYCANGHTFQAKRFNRRAHCAICTD
ζ PEQPGMPCPGEDKSIYRRGARRWRKLYRANGHLFQAKRFNRGAYCGQCSE
ι RIWGLGRQGYKCINCKLLVHKKCHKLVTIECGRH..SLPQEPVMPMDQSS 200
λ RIWGLGRQGYKCINCKLLVHKKCHKLVTIECGRH..SLPPEPMMPMDQ.T
ζ RIWGLSRQGYRCINCKLLVHKRCHVLVPLTCRRHMDSVMPSQEPPVDGKN
ι ....MHSDHAQTVIPYNPSS..HESLDQVGEE.KEAMNTRESGKASSSLG 250
λ ....MHPDHTQTVIPYNPSS..HESLDQVGEE.KEAMNTRESGKASSSLG
ζ DGVDLPSEETDG.IAYISSSRKHDNIKDDSEDLKPVIDGVDGIKISQGLG
ι LQDFDLLRVIGRGSYAKVLLVRLKKTDRIYAMKVVKKELVNDDEDIDWVQ 300
λ LQDFDLLRVIGRGSYAKVLLVRLKKTDRIYAMKVVKKELVNDDEDIDWVQ
ζ LQDFDLIRVIGRGSYAKVLLVRLKKNDQIYAMKVVKKELVHDDEDIDWVQ
ι TEKHVFEQASNHPFLVGLHSCFQTESRLFFVIEYVNGGDLMFHMQRQRKL 350
λ TEKHVFEQASNHPFLVGLHSCFQTESRLFFVIEYVNGGDLMFHMQRQRKL
ζ TEKHVFEQASSNPFLVGLHSCFQTTSRLFLVIEYVNGGDLMFHMQRQRKL
ι PEEHARFYSAEISLALNYLHERGIIYRDLKLDNVLLDSEGHIKLTDYGMC 400
λ PEEHARFYSAEISLALNYLHERGIIYRDLKLDNVLLDSEGHIKLTDYGMC
ζ PEEHARFYAAEICIALNFLHERGIIYRDLKLDNVLLDADGHIKLTDYGMC
ι KEGLRPGDTTSTFCGTPNYIAPEILRGEDYGFSVDWWALGVLMFEMMAGR 450
λ KEGLRPGDTTSTFCGTPNYIAPEILRGEDYGFSVDWWALGVLMFEMMAGR
ζ KEGLGPGDTTSTFCGTPNYIAPEILRGEEYGFSVDWWALGVLMFEMMAGR
ι SPFDIVGSSDNPDQNTEDYLFQVILEKQIRIPRSLSVKAASVLKSFLNKD 500
λ SPFDIVGSSDNPDQNTEDYLFQVILEKQIRIPRSLSVKAASVLKSFLNKD
ζ SPFDIIT..DNPDMNTEDYLFQVILEKPIRIPRFLSVKASHVLKGFLNKD
ι PKERLGCHPQTGFADIQGHPFFRNVDWDMMEQKQVVPPFKPNISGEFGLD 550
λ PKERLGCHPQTGFADIQGHPFFRNVDWDMMEQKQVVPPFKPNISGEFGLD
ζ PKERLGCRPQTGFSDIKSHAFFRSIDWDLLEKKQTLPPFQPQITDDYGLD
ι NFDSQFTNEPVQLTPDDDDIVRKIDQSEFEGFEYINPLLMSAEECV 596
λ NFDSQFTNEPVQLTPDDDDIVRKIDQSEFEGFEYINPLLMSAEECV
ζ NFDTQFTSEPVQLTPDDEDVIKRIDQSEFEGFEYINPLLLSAEESV

Figure 5.4 Comparison of nucleotide sequences of PCR primers I1 and I2 with the sequences from PKC ι and λ to which these primers are designed to bind.

The sequence of the oligonucleotide primers I1 and I2, designed to amplify PK
are shown aligned with the nucleotide sequence for human PKC ι and the mo
homologue PKC λ as reported in the GenBank™ data base. Bars indicate identical nucleotides and alternative nucleotides in brackets represent redundancies in the primer sequence. The identity of the each sequence is shown on the left while the nucleotide numbers are shown on the right.

Figure 5.4

a) Oligonucleotide primer I1

```
pkc λ      TGGGACACGGICGCGIGCGG
           | | ||||||||||| ||||
I1         tcicacacigtcgciggcgg 20
           ||||||||||||||||||||
pkc ι      TCCCACACGGICGCAGGCGG 23
```

b) Oligonucleotide primer I2

```
pkc λ      ATGCATCCAGACCA   C ACACAGAC
           |||||| | |||||    |  |||||||
I2         atgcattctgacca(t/c)gcacagac  1
           |||||||||||||| |   ||||||||
pkc ι      ATGCATTCTGACCA T   GCACAGAC 617
```

Figure 5.5 Agarose gel electrophoresis of the products from reverse transcriptase PCR amplification using primers Z3 and Z4.

10 μl of the reaction mixture after PCR amplification with oligonucleotide primers Z3 and Z4 of RNA from the αT3-1 pituitary gonadotroph cell line was analysed by electrophoresis through a 1% agarose gel containing 0.5 mg/ml ethidium bromide. A strong band was visible at approximately 632 bp (as indicated by the arrow), the predicted size for PKC ζ, but no larger fragments were detected. Sizes of the molecular weight markers are indicated in base pairs.

Figure 5.5

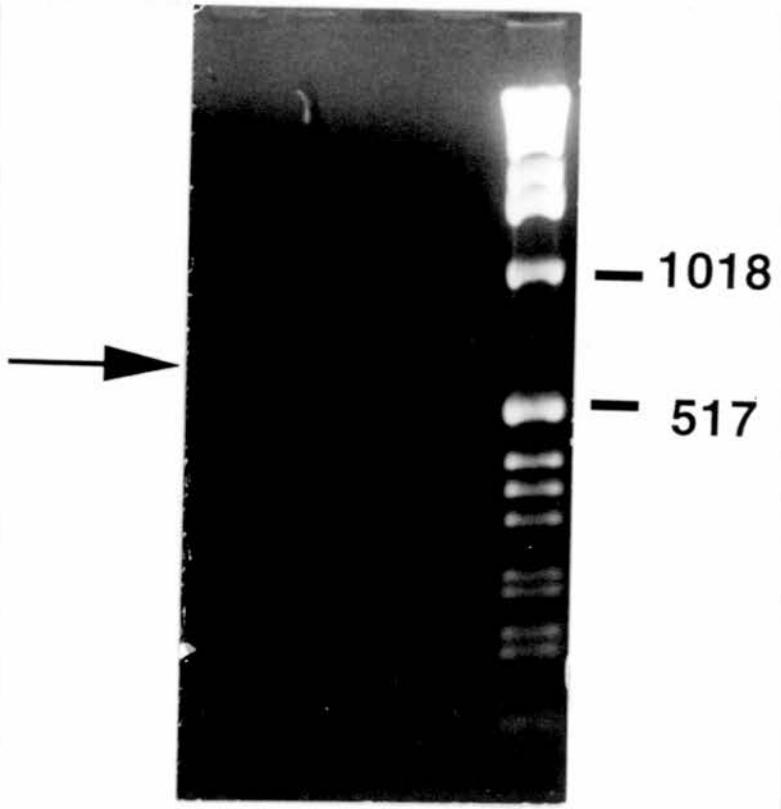


Figure 5.6 DNA sequencing analysis of the PCR product from PCR amplification using primers Z3 and Z4.

The identity of the product from αT3-1 cells of PCR amplification using the PKC ζ specific primers Z3 and Z4 was confirmed by DNA sequencing analysis. Asymmetric PCR amplification of the original PCR product was conducted as described in Section 5.2, resulting in a single-stranded product. This was then used as the template for DNA sequencing by the dideoxy method, using Z3, the limiting PCR primer as the primer for the sequencing reaction. The resulting sequence is shown aligned with the nucleotide sequence for mouse PKC ζ as reported in the GenBank™ data base. The identity of the each sequence is shown on the left while the nucleotides are numbered on the right. The location of the Z3 PCR/sequencing primer is indicated by the shaded box ▨.

Figure 5.6

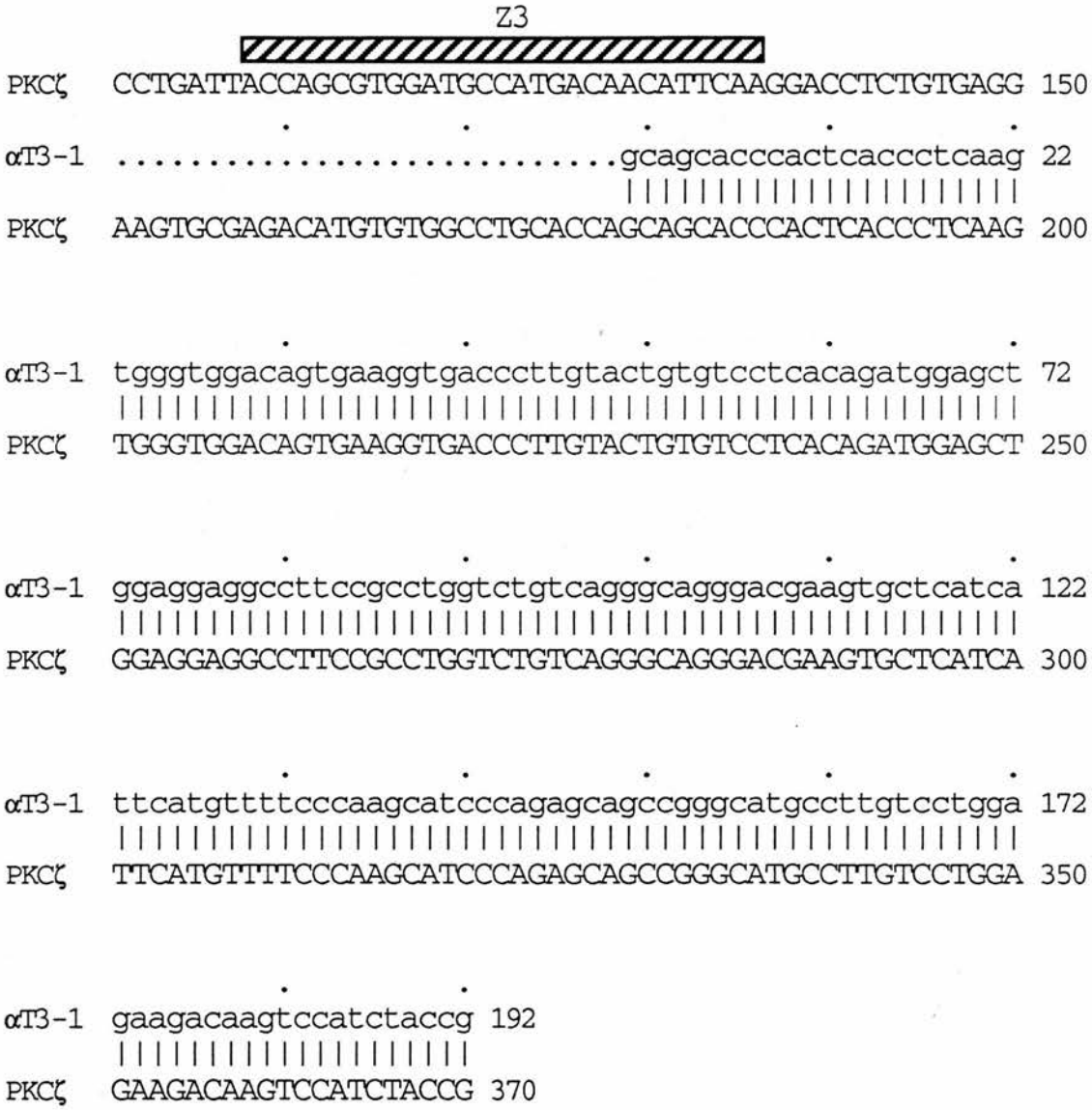
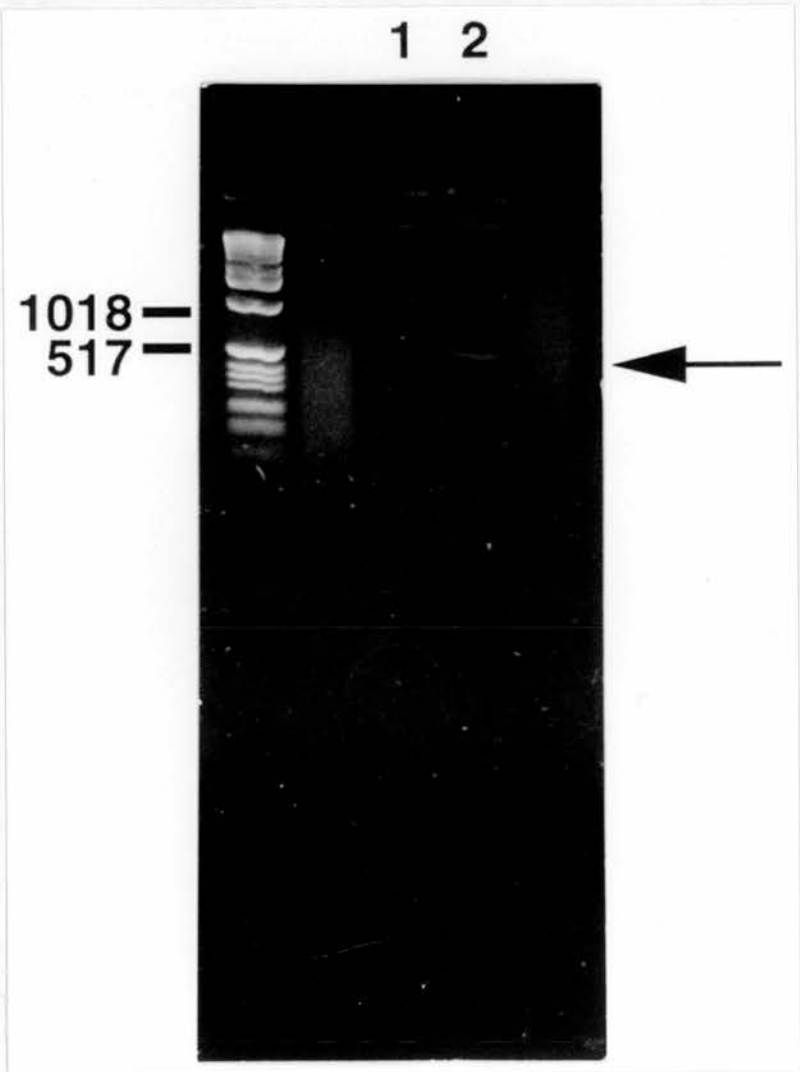

Z3
PKCζ CCTGATTACCAGCGTGGATGCCATGACAACATTCAAGGACCTCTGTGAGG 150
αT3-1 .....gcagcacccactcaccctcaag 22
PKCζ AAGTGCGAGACATGTGTGGCCTGCACCAGCAGCACCCACTCACCCTCAAG 200
αT3-1 tgggtggacagtgaaggtgacccttgtactgtgtcctcacagatggagct 72
PKCζ TGGGTGGACAGTGAAGGTGACCCTTGTACTGTGTCCTCACAGATGGAGCT 250
αT3-1 ggaggaggccttccgcctggtctgtcagggcagggacgaagtgctcatca 122
PKCζ GGAGGAGGCCTTCCGCCTGGTCTGTCAGGGCAGGGACGAAGTGCTCATCA 300
αT3-1 ttcatgttttcccaagcatcccagagcagccgggcatgccttgtcctgga 172
PKCζ TTCATGTTTTCCCAAGCATCCCAGAGCAGCCGGGCATGCCTTGTCCTGGA 350
αT3-1 gaagacaagtccatctaccg 192
PKCζ GAAGACAAGTCCATCTACCG 370

Figure 5.7 Agarose gel electrophoresis of the products from reverse transcriptase PCR amplification of rat kidney RNA.
10 μl of the reaction mixture after PCR amplification with oligonucleotide primers Z3 and Z4 (Lane 2) and I1 and I2 (Lane 1) was analysed by electrophoresis through a 1% agarose gel containing 0.5 mg/ml ethidium bromide. A strong band was visible at approximately 632 bp (as indicated by the arrow), the predicted size for PKC ζ, following amplification using Z3 and Z4 (Lane 2) but there was no product visible from amplification using I1 and I2 (Lane 1). Sizes of the molecular weight markers are indicated in base pairs.

Figure 5.7



CHAPTER 6

HOMOLOGY CLONING EXPERIMENTS DESIGNED TO ISOLATE cDNAs ENCODING THE H7-RESISTANT PKC

6.1 INTRODUCTION

After the initial discovery of PKC and its activation in response to diacylglycerol and Ca^{2+}, PKC was soon found to play a role in the induction of cellular responses by a variety of ligand-receptor systems (Berridge and Irvine, 1984; Nishizuka, 1984a) and in regulation of cellular responsiveness to external stimuli (Hunter et al., 1985). However, it remained unclear how a single molecule could mediate and control such a diverse variety of signaling pathways (Coussens et al., 1986). It was only when PKC was cloned that evidence emerged for a PKC gene family (Coussens et al., 1986) and the true extent of the heterogeneity of this family is only now being revealed.

Protein kinase C was initially cloned following isolation from bovine brain using a number of protein purification techniques (Parker et al., 1984). Partial amino acid sequence was obtained from tryptic peptide fragments and regions of this sequence were selected for use as oligonucleotide probes to screen a cDNA library, in order to obtain the full length clone and thus the full cDNA sequence of PKC (Parker et al., 1986). This soon lead to the identification of other related cDNAs, thus PKC α, β and γ had been cloned (Coussens et al., 1986). A number of groups simultaneously cloned Ca^{2+}-dependent PKCs (Knopf et al., 1986; Parker et al., 1986) and PKC became recognised as a gene family of closely related enzymes. This allowed comparison of the structure of these PKCs and the conserved regions of the protein could be identified (Coussens et al., 1986). The occurrence of further heterogeneity, as a result of alternative splicing, also became evident with the isolation of cDNA clones PKC βI and II, which differed only in the carboxyl-terminal regions of 50 and 52 amino acid residues respectively (Ono et al., 1986). As isoforms were cloned from a

number of different species, it became clear that the sequences of the PKCs were highly conserved between mammalian species, even in the regions that varied between isoforms, suggesting that all regions of the primary structure may be important in the function of the individual PKC isoforms (Kubo et al., 1987).

Although the existence of a phorbol ester receptor with Ca^{2+}-independent protein kinase activity had reported earlier (Shoyab and Boaze, 1984), it was only later that sequences of the Ca^{2+}-independent PKC isoforms were identified. The method that had successfully isolated PKC β and γ was once again employed, using fragments of the known PKC sequences as hybridisation probes to identify further PKC-related clones from a cDNA library. The structures of PKC δ, ε and ζ were discovered in this way and they were identified as being related to the previously known PKC family members, though they differed in their regulatory domains (Ono et al., 1988).

Protein kinase C θ was the first isoform to be cloned using a different approach. Three groups independently reported cloning this isoform, one using the strategy previously successful in isolating δ, ε and ζ (Osada et al., 1992), which had also revealed PKC η (Osada et al., 1990), while the other two groups employed the Polymerase Chain Reaction (PCR) technique (Baier et al., 1993; Chang et al., 1993). Both groups aimed to identify PKC family members present in human blood cells and used degenerate oligonucleotide primers to regions conserved between the PKC isoforms. This should result in amplification of the region between the two primers, from all the PKC sequences that are present in these cells. DNA sequencing of the resulting products allowed identification of a novel PKC-like sequence, and the corresponding full length cDNA was obtained either by using the fragment as a probe to screen a cDNA library or by anchored

PCR amplification of the 3' and 5' ends. The two members of the PKC family that have since been identified, PKC ι and μ, were also cloned using a PCR-based strategy (Pfizenmaier et al., 1993; Selbie et al., 1993).

The evidence presented in Chapter 4 suggested that the H7-resistant PKC detected in anterior pituitary cytosol is a novel PKC isoform, as post-translational modifications of known isoforms seem unlikely to account for the apparent molecular mass of 130 kDa, 40-50 kDa larger than the well characterised isoforms. As the amount of this protein present in the pituitary is low, it was decided to attempt to clone this PKC, rather than to isolate it by protein purification techniques. In view of the number of recent successes using PCR to clone isoforms, this method was selected. Both total RNA extracted from pituitary tissue and a pituitary cDNA library were used as a template for amplification. Total RNA from αT3-1 clonal pituitary cell line that had been treated with oestrogen was also used. These cells have also been shown to contain the H7-resistant PKC (Chapter 4 and (Simpson et al., 1994)) and, as this PKC has been shown to be induced in pituitaries by oestrogen pretreatment (Thomson et al., 1993a), it is possible that these cells may represent a good source from which to clone this PKC.

6.2 Specific Methodology

Total RNA was prepared as described in Section 2.9 from either eight rat pituitaries or two 75 cm^2 flasks of αT3-1 cells, which had been split to 50% confluency two days prior to treatment for 24 hours with 10 nM oestrogen. A pituitary cDNA library was constructed by Clontech Laboratories Inc. (Palo Alto, CA, USA) in the bacteriophage vector λZapII, from mRNA from male and female anterior pituitaries. PCR was conducted as described in Sections 2.10.1 (PCR from cDNA libraries) and 2.10.2 (reverse transcriptase PCR), using degenerate oligonucleotide primers

designed as described in Section 6.3. Initial PCR conditions with the primers 592K and 593K were as follows:Step 1, denaturation at 94°C for 1 min; Step 2, annealing at 55°C for 1 min; Step 3, extension at 72°C for 1 min. This cycle was repeated 5 times followed by 35 cycles during which the annealing step was conducted at 60°C, and a final cycle, annealing at 60°C, during which the extension step was continued for 8 min. In subsequent experiments the annealing temperature for the first five cycles was varied between 45 and 55°C. Using primers PC3 and 593K, initial PCR conditions were as follows: Step 1, denaturation at 94°C for 1 min; Step 2, annealing at 60°C for 1 min; Step 3, extension at 72°C for 1 min. This cycle was repeated 5 times followed by 35 cycles during which the annealing step was conducted at 68°C, and a final cycle, annealing at 68°C, during which the extension step was continued for 8 min. For subsequent experiments using the same pair of primers, the annealing temperature was decreased to 55°C or 45°C for the first 5 cycles and 60°C or 55°C for the other 35 cycles. One tenth of the amplified products was analysed by agarose gel electrophoresis (Section 2.18.2) and DNA fragments were purified using one of the methods described in Section 2.11. Products were ligated into the pBluescript, pGEM4z or pGEM-T vectors (Promega) and propagated in the DH5α strain of *E. coli*. Mini prep DNA was prepared from colonies as described in Section 2.15 and an aliquot (approximately one fifth) was analysed by restriction digestion. Sequencing analysis was conducted as described in Section 2.17.

6.3 RESULTS

PCR amplification of 293 base pair fragments of cDNAs encoding PKC isoforms

In order to identify novel genes potentially related to the PKC gene family that are expressed in the anterior pituitary and may represent the H7-resistant kinase, a PCR based strategy was used. Initially two degenerate oligonucleotide primers were designed to regions in the catalytic domain which are conserved between the PKC isoforms. Potential primer sequences were analysed for complementary nucleotide sequences that might allow dimerisation of the primers or self-annealing, the occurrence of which would decrease the efficiency of amplification of PKC-related products. The chosen primers sequences, 592K and 593K (shown in Table 6.1) correspond to the amino acid sequences LFFVMEYVNGGDLMF (residues 412-426 in PKC α) and TFCGTPDYIAPEI (residues 497-509 in PKC α) respectively. Their positions in the catalytic domain and the homology between the known PKCs in these regions are shown in Figure 6.1. PCR amplification of cDNA templates, prepared from pituitary RNA, resulted in products of the expected 293 bp size (Figure 6.2). The primer sequences contained restriction sites for the enzymes TaqI (592K) and XhoI (593K), which were initially used to ligate the products into pBluescript. After transformation into *E. coli*, DNA was prepared from 10 colonies and restriction analysis was conducted to investigate the possible identity of the PCR fragments they contained. DNA samples were digested with the endonucleases, NcoI and SalI or with these two enzymes as well as XbaI and the results compared. As shown in Figure 6.3a, NcoI and SalI cut the plasmid in the polylinker, while XbaI cleaves DNA at a sequence that is specific to the PCR product resulting from amplification of PKCε. This

identified all 10 clones as fragments of PKC ε, an example of which is shown in Figure 6.3c. This was confirmed by sequencing analysis (Figure 6.4).

In view of the initial PCR only resulting in amplification of PKC ε, less stringent conditions were used, with a lower temperature at the annealing step of the PCR cycle, to allow amplification of other sequences. The resulting products of the expected size were ligated into the pGEM4z vector and, following transformation, DNA from sixteen of the resulting colonies was prepared. Restriction digestion and sequencing analysis was conducted as previously described. This identified three of the colonies as containing PCR fragments encoding PKC ε, the other 13 corresponding to three sequences that did not show significant homology to any of the known PKC sequences, or to any of the sequences in the GenBank™ and EMBL data bases.

A third PCR amplification using these primers was conducted under conditions of intermediate stringency in an attempt to reduce the amplification of unrelated sequences but still allow amplification of PKC-like sequences, other than PKC ε. The resulting PCR fragments were ligated into the pGEM-T vector and, following transformation, DNA was prepared from 59 colonies. Ten of these were identified as containing fragments encoding PKC ζ, by restriction analysis using the endonucleases RsaI, which linearises the pGEM-T vector, and HindIII, which cleaves samples at a site specific to PKC ζ, as shown in Figure 6.5. This identification was confirmed by sequencing analysis (Figure 6.6). The sequence of one clone, shown by restriction analysis to encode neither PKC ε or ζ, was found to correspond to PKC α (Figure 6.7). Of the other 48 clones, 32 represented PKC ε and the sequences of the other 16 showed no homology to any of the known PKC isoforms or any other sequence in the GenBank™ and EMBL data bases.

PCR amplification of 494 base pair fragments of cDNAs encoding PKC isoforms

As the sense primer 592K would be unlikely to anneal to PKC θ (the sequence of which had been published since the design of this primer) and therefore may be unable to recognise other PKC-related sequences, a new sense primer, PC3, was designed. The sequence of this primer (Table 6.1) corresponds to the amino acids LGKGSFGKV (residues 333-342 in PKC α) within the ATP binding site, as shown in Figure 6.1. PCR amplification from the pituitary cDNA library was conducted as described in Sections 2.10.1 and 6.2 using PC3 and 593K, the original antisense primer. This produced a fragment of the expected size (494 bp)(Figure 6.8). This was ligated into the pGEM-T vector and, following transformation, DNA prepared from 12 colonies was analysed by restriction digestion. This identified all clones as PKC ε (Figure 6.9) and this was confirmed by sequencing analysis (Figure 6.10). PCR amplification was repeated using these primers under less stringent cycling conditions and 31 of the resulting clones were analysed as previously, one of which encoded PKC α and the remaining 30 corresponded to PKC ε. Some of the DNA resulting from this amplification was also digested with the restriction enzyme XbaI, which cleaves sequences encoding PKC ε but not the other PKC isoforms. The uncut fragments were separated from cut ones by agarose gel electrophoresis and gel purification, and subsequently ligated into the pGEM-T vector. However the DNA from five of the colonies resulting from transformation, when analysed by sequencing were all identified as encoding PKC ε.

PCR amplification from oestrogen-treated αT-1 cells

As oestrogen treatment has been shown to induce the H7-resistant PKC involved in LH secretion from anterior pituitaries (Thomson et al.,

1993a), it was decided to see if pretreatment may favour PCR amplification of a novel PKC-like sequence. Therefore αT3-1 cells were pretreated with oestrogen for 24 hours prior to RNA extraction. The resulting total RNA was used as the template for reverse transcriptase PCR using primers 592K and 593K. Of the 10 clones analysed by sequencing analysis, 6 were identified as PKC ε and the remaining 4 were found to encode PKC ζ.

6.4 Discussion

The original pair of primers, 592K and 593K, correspond to sequences located in the C3 region of the catalytic domain, which are shared by PKC isoforms α-ι (Figure 6.1) but are not conserved in other protein kinases. The resulting 293 bp fragments correspond to a region within which amino acid identity amongst the known PKC sequences is approximately 63%, while the corresponding homology levels of known PKC genes with their nearest relative, cAMP-dependent protein kinase type α (PKA α), is <41%. Thus, although this region is broadly conserved among members of the PKC gene family, isoforms vary sufficiently to allow amplified sequences to be identified as potentially novel members of the PKC family.

Amplification using these primers in each case resulted in products of the expected size (293 bp) as calculated from the sequences previously published for the rat PKC isoforms α-ι (Selbie et al., 1993). By varying the annealing temperatures of the PCR cycle, the selection of amplified products was altered. Initial conditions resulted in the amplification of only cDNA fragments encoding PKC ε, as determined by restriction and sequencing analysis (Figure 6.3 and 6.4). This suggests that either PKC ε may be the most abundant PKC isoform in the anterior pituitary, or the primers used favour amplification of this isoform over the other PKCs

present in anterior pituitary cells. Therefore the stringency of the PCR cycling conditions was varied in an attempt to optimise amplification of a variety of PKC-related sequences, while minimising amplification of unrelated products. This resulted in amplification of sequences that were identified as PKC ζ in addition to PKC ε, as well as one sequence encoding PKC α. The primer 592K was designed to favour amplification of the Ca^{2+}-independent PKC isoforms, as the nucleotides at the 3' end of this oligonucleotide encode phenylalanine, which only occurs in this position of the sequences encoding the nPKCs and PKC ζ and ι (Selbie et al., 1993). Thus this explains the low incidence of PKC α and absence of PKC β encoding sequences, both of these isoforms being present in anterior pituitary (Figure 4.6), in the amplified products.

Initial PCR experiments failed to amplify the other nPKCs that have been shown to be present in the anterior pituitary (PKC δ and θ)(Figure 4.6). This may be due to the primers favouring amplification of PKC ε and ζ. The upstream primer 592K is unlikely to anneal to PKC θ, in addition to the Ca^{2+}-dependent PKC isoforms, as this isoform also lacks the phenylalanine encoded at the 3' end of this primer sequence (Baier et al., 1993; Chang et al., 1993). As this means that it is possible that this primer may not amplify other PKC-related sequences, another sense primer, PC3$_4$ was designed as an alternative to 592K for use with the antisense primer 593K. This primer (P3) corresponds to a sequence at the start of the catalytic domain within the ATP binding site, which is highly conserved between PKC α-η (Figure 6.1). PKC ζ and ι vary slightly from the other PKC isoforms in this region so it is possible that this primer may not amplify these isoforms. However both PKC ζ and ι, unlike the H7-resistant PKC, are not stimulated by phorbol esters and primers specific for these isoforms had failed to identify in anterior

pituitary cells any potentially related PKCs which may be extended in the phorbol binding domain (Chapter 5).

Amplification using the primers PC3 and 593K resulted in a product of 494 bp, the size predicted from the sequences published for PKC α-ι (Selbie et al., 1993). Cloning of this product resulted predominantly in clones containing the sequence encoding PKC ε (Figure 6.10), while one contained a sequence encoding PKC α. The lack of PKC ζ sequence suggests that, as previously proposed, the primer PC3 may not be able to bind to sequences encoding this isoform. Although the conditions of PCR cycling using this pair of primers were varied, it is possible that they were still too stringent to allow amplification of other PKC-related sequences. Alternatively PKC ε and ζ may be expressed at higher levels in anterior pituitary cells than the other PKC isoforms that are known to be present (PKC α, β, δ and θ) which would mean that the probability of amplifying fragments encoding these predominant isoforms would be much greater than that for amplification of the other less abundant isoforms. This is supported by evidence from Northern blots using oligonucleotide probes directed against unique sequences in PKC δ-ζ, which detected PKC ε and ζ but only small amounts of PKC δ in anterior pituitary cells (E. Lutz, A. Ison and R. Mitchell, unpublished observations). In an attempt to eliminate sequences encoding PKC ε, the product of PCR amplification with these primers was incubated with the restriction endonuclease XbaI prior to ligation, as this enzyme cleaves a sequence that is present in the amplified fragment of PKC ε but not the other PKC isoforms. However, the sequences that were obtained from ligating into the pGEM-T vector only the PCR products that remained uncleaved following digestion were subsequently identified as PKC ε, suggesting that the digestion of the DNA was incomplete.

To try to favour amplification of novel PKC-like sequences that might correspond to the H7-resistant kinase, oestrogen-treated αT3-1 cell RNA was used as the template for PCR amplification, using primers 592K and 593K. Oestrogen has been shown to induce the H7-resistant PKC that is involved in LH release from rat pituitaries, so it was hoped that RNA from this pituitary cell line derived from LH secreting cells treated with oestrogen may be enriched in this kinase and thereby increase the chances of amplifying a novel PKC-related sequence. However, oestrogen has also been shown to increase the total PKC content of all types of pituitary cells (Drouva et al., 1990), so it is likely to stimulate expression of other PKC isoforms, in addition to the H7-resistant kinase. This may explain the failure of this approach to selectively amplify sequences other than those encoding PKC ε and ζ.

There are still a number of approaches yet to be explored that may result in amplification of a novel PKC-related sequence. It is possible that the antisense primer used in these experiments is biased towards amplification of PKC ε rather than other sequences, so an alternative primer could be designed for PCR with either of the sense primers used previously. The use of two pairs of nested primers may also be considered, as this was the strategy that resulted in the cloning of PKC θ (Chang et al., 1993). Using this approach, a first round of amplification could be conducted at low stringency, using one set of primers corresponding to regions that are conserved between PKC isoforms, and the products could then be used as the template for a second round of amplification, using different primers. The second pair of primers bind to conserved sequences that are contained within the fragment amplified in the initial PCR reaction. This means that sequences unrelated to PKC that may have been amplified in the first round of PCR, are unlikely to be amplified in the second round, as they should not

contain the sites to which the second pair of primers bind. This technique therefore avoids the problem of amplification of unrelated sequences of unknown identity, which occurred in these experiments when the stringency of the PCR conditions was reduced, as only PKC-related sequences would be amplified on the second round of amplification. The resulting clones could be screened by colony hybridisation, using a probe that was designed to hybridise to PKC ε, which would enable analysis only of clones thought to contain sequences other than PKC ε.

Evidence presented in Chapter 4 suggested that the H7-resistant PKC may correspond to a 130 kDa protein. This molecular mass is much higher that those reported for PKC α-ι (65-90 kDa), which suggests that this PKC could be more closely related to PKC μ, which has a molecular mass of 115 kDa(Pfizenmaier et al., 1993). PKC μ has been assigned to the PKC family as it shows significant homologies in both the regulatory and catalytic domains, though it is more distantly related than the other PKCs. The amino terminus of PKC μ contains a putative signal peptide and transmembrane domain which is not present in any of the other PKC isoforms. The catalytic domain of PKC μ shows 25-31% amino acid identity with the other Ca^{2+}-independent PKCs while these isoforms show 45-75% homology in this region. Analysis of the sequence of PKC μ, which has only been recently published and was not available at the time of designing the PCR primers used in this study, suggests that this isoform may not be amplified even if present in anterior pituitary using these primers. Therefore the failure of these experiments to identify a novel sequence representing the H7-resistant kinase may conceivably be due to similarity of this kinase to PKC μ, rather than the other PKC isoforms. PKC μ, however, is not activated by phorbol esters, unlike the H7-resistant kinase, although it differs from the other phorbol unresponsive PKCs as it contains two of the cysteine-rich

regions thought to be responsible for phorbol binding. Thus, while the H7-resistant kinase may resemble PKC μ, it is unlikely to share high homology with this isoform in the C1 domain of the regulatory subunit. However designing primers based on the amino acid residues in the catalytic domain that are conserved between PKC μ and the other PKCs may increase the probability of amplifying a novel PKC-related sequence.

Table 6.1 PKC-specific oligonucleotide primers used in this study.

Primer	Sequence	Nucleotide positions
592K (sense)	5'-(C/T)TIGTCATGGAGT(A/T)(T/C) GTCAACGGGGGIGACCTCAT GTTC-3'	1456-1497
593K (antisense)	5'-CAGGATCTCIGGGGCGAT(A/G) TAGT(T/C)A(A/G)GGTICC-3'	1708-1749
PC3 (sense)	5'-TIGGIAA(A/G)GGIAG(C/T)TT(C/T) GG(C/G)AAGGT-3'	1242-1267

Details regarding the specific use of each oligonucleotide are given in Sections 2.10 and 6.2. Redundancies were inserted where PKC isoforms varied in their codon usage or amino acid sequence. Nucleotide positions are derived from the cDNA sequence of PKC α.

Figure 6.1 Comparison of amino acid sequence of the catalytic domain of PKC isoforms α-ι.

The amino acid sequence of the C3/C4 regions (catalytic domain) for PKC α-ι, either rat or human are shown and regions that are identical in at least 60% of the aligned sequences are boxed. Individual PKC isoforms are denoted by the appropriate Greek letter on the left, and the amino acid residues are numbered on the right. The positions of the oligonucleotides used as primers for PCR are indicated by the shaded boxes; ▨ 592K, ▧ 593K, ▨ PC3. The bar indicates the ATP binding domain.

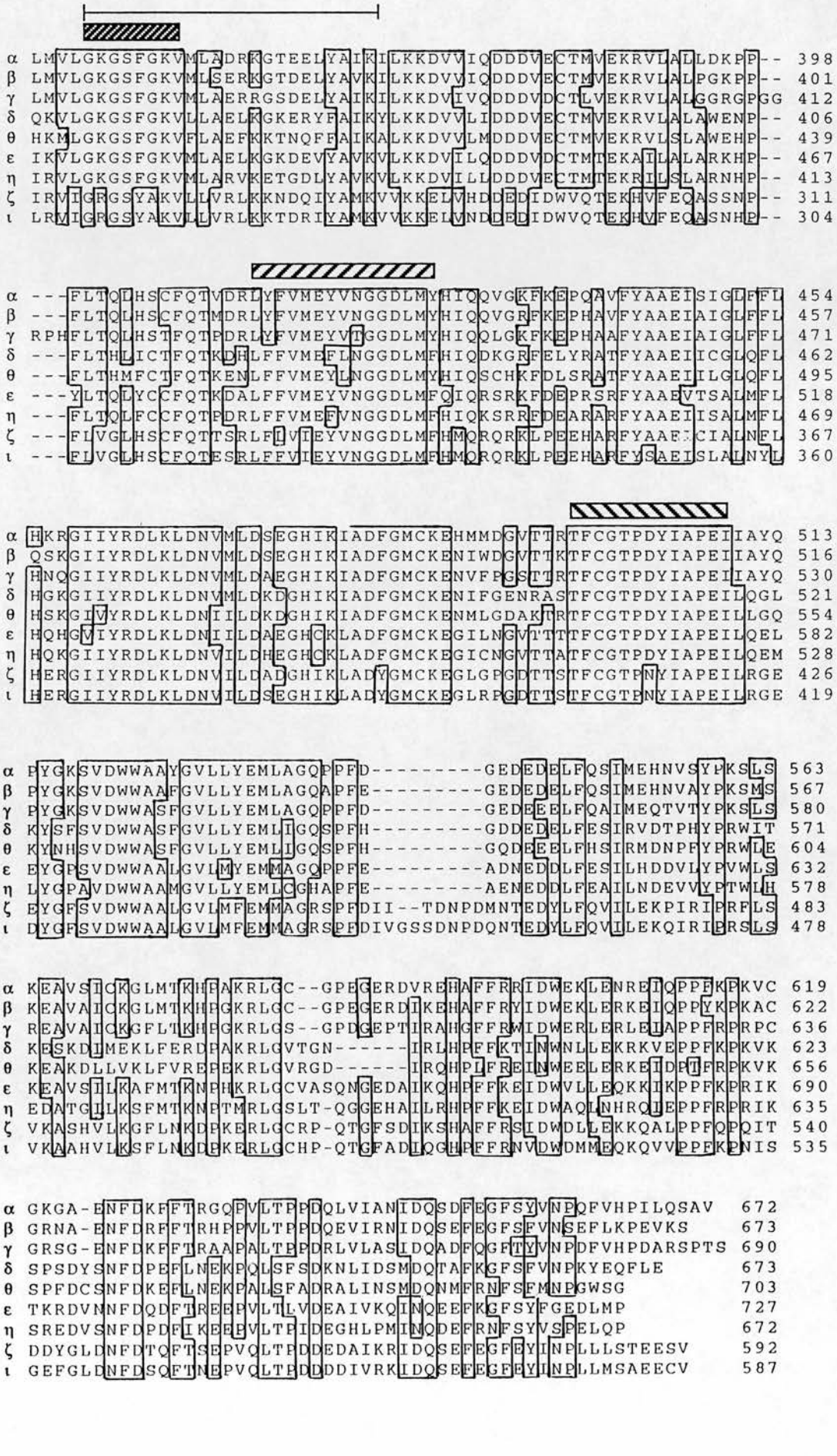


Figure 6.2 Agarose gel electrophoresis of the products from PCR amplification of reverse transcribed RNA encoding PKC isoforms expressed in rat anterior pituitary gland.

10 μl of the reaction mixture after PCR amplification with oligonucleotide primers 592K and 593K was analysed by electrophoresis through a 1% agarose gel containing 0.5 mg/ml ethidium bromide. A band was visible at approximately 300 bp (as indicated by the arrow), the predicted size for PKC isoforms α-ι. Sizes of the molecular weight markers are indicated in base pairs.

Figure 6.2

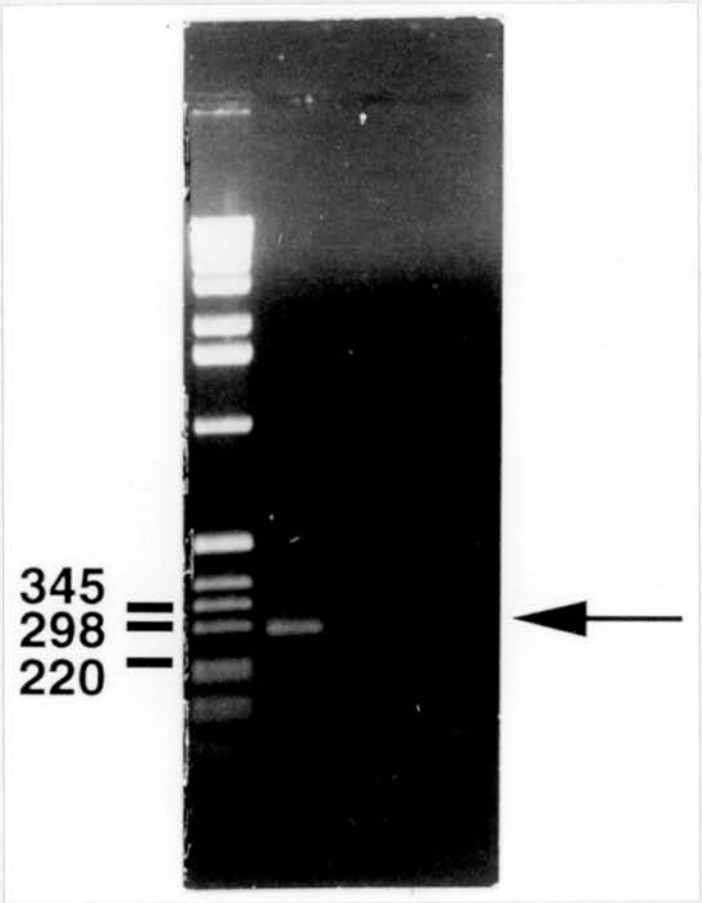
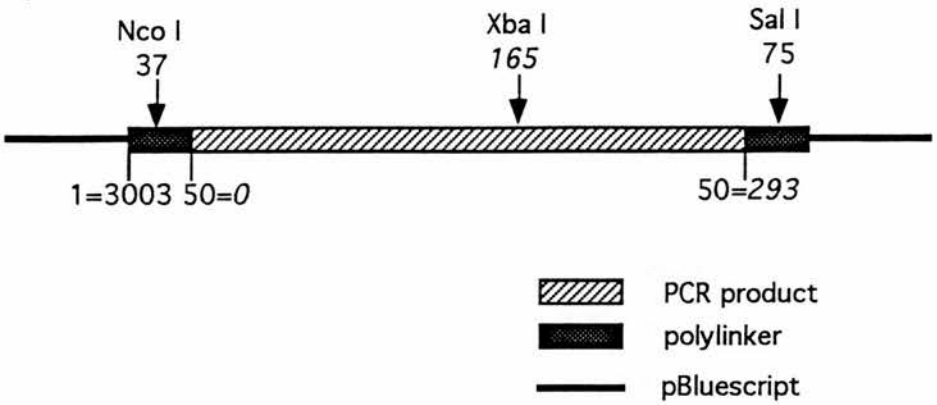


Figure 6.3 Identification of sequences encoding PKC ε by restriction analysis.

Products of PCR amplification were ligated into a plasmid vector and transformed into *E coli*. An aliquot of the DNA prepared from each colony selected was analysed by digestion with the restriction endonucleases, NcoI, SalI and XbaI in the One-for-All restriction enzyme buffer (1.5x)(Promega). a) shows the location of restriction sites for these enzymes in a construct containing PCR fragment encoding PKC ε. Numbers refer to positions in the cloning vector, or in the amplified PCR product (in italics). b) shows the expected size of the fragments obtained from cleavage of a clone containing the sequence encoding PKC ε. c) Agarose gel electrophoresis of typical restriction digests showing a PKC ε-containing plasmid (lane 2) and plasmid containing a PCR product with a different sequence (lane 1). The top arrow indicates the fragment corresponding to the cloning vector and the other arrows show the insert either encoding PKC ε (bottom) or a different sequence (middle). Sizes of the DNA molecular weight markers are shown in base pairs.

Figure 6.3

a)



b)

Fragment	Expected size (base pairs)
Vector (SalI-NcoI)	2965
Insert (NcoI-XbaI)	178 (141)
Insert (XbaI-SalI)	143 (176)

c)

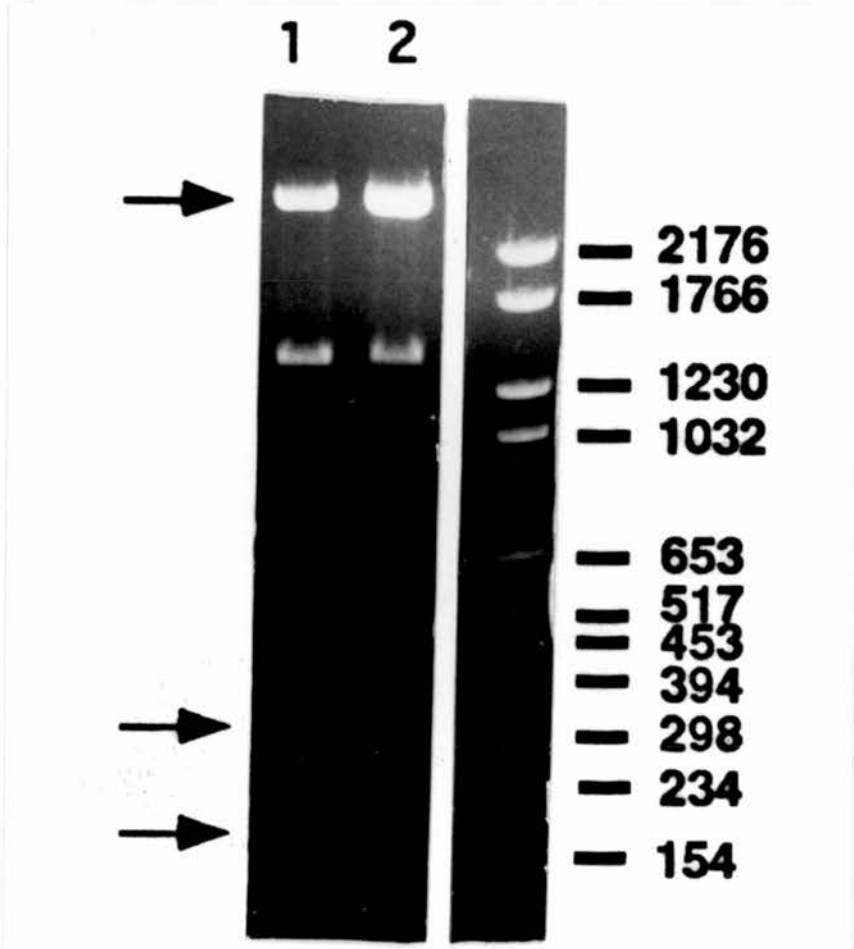


Figure 6.4 DNA sequencing analysis of a clone containing a PCR product encoding PKC ε.

The identity of some of the colonies indicated by restriction analysis as containing fragments encoding PKC ε was confirmed by DNA sequencing analysis using the dideoxy method. One such sequence, 4r is shown aligned with the nucleotide sequence for rat PKC ε as reported in the GenBank™ data base. Bars indicate identical nucleotides and the site of cleavage by XbaI, the endonuclease used in restriction analysis, is shown by the arrow. The identity of the each sequence is shown on the left while the nucleotides are numbered on the right. Sequences representing the PCR primers are indicated by the shaded boxes; ▨ 592K, ▧ 593K.

Figure 6.4

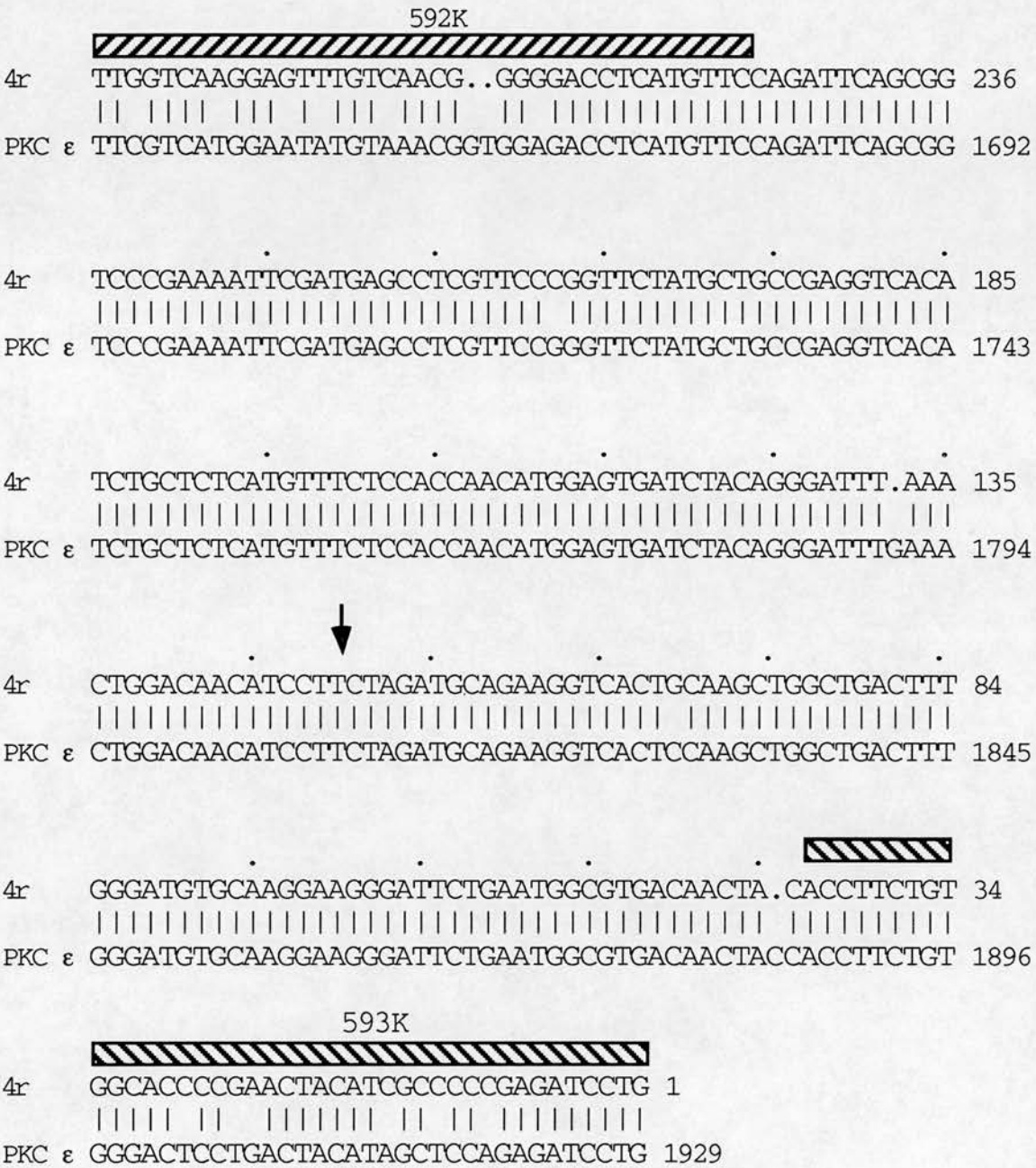
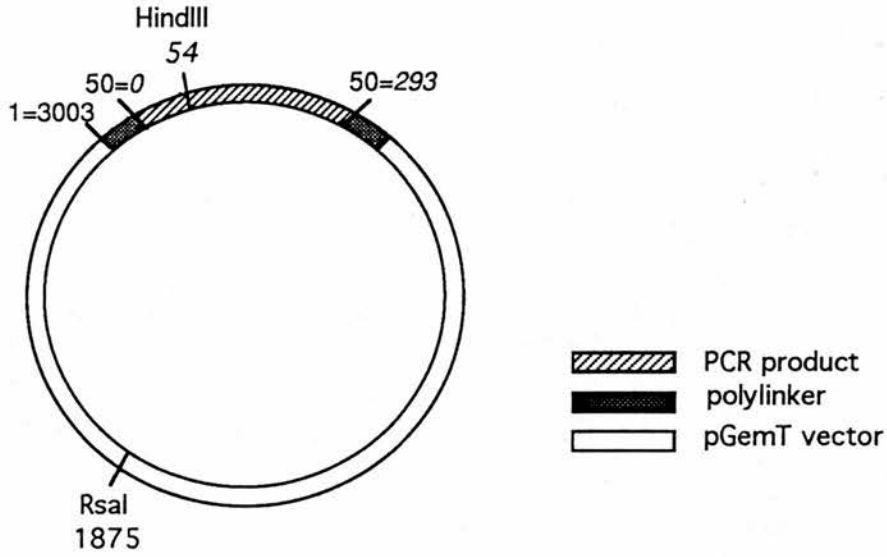

592K
4r TTGGTCAAGGAGTTTGTCAACG..GGGGACCTCATGTTCCAGATTCAGCGG 236
PKC ε TTCGTCATGGAATATGTAAACGGTGGAGACCTCATGTTCCAGATTCAGCGG 1692
4r TCCCGAAAATTCGATGAGCCTCGTTCCCGGTTCTATGCTGCCGAGGTCACA 185
PKC ε TCCCGAAAATTCGATGAGCCTCGTTCCGGGTTCTATGCTGCCGAGGTCACA 1743
4r TCTGCTCTCATGTTTCTCCACCAACATGGAGTGATCTACAGGGATTT.AAA 135
PKC ε TCTGCTCTCATGTTTCTCCACCAACATGGAGTGATCTACAGGGATTTGAAA 1794
4r CTGGACAACATCCTTCTAGATGCAGAAGGTCACTGCAAGCTGGCTGACTTT 84
PKC ε CTGGACAACATCCTTCTAGATGCAGAAGGTCACTCCAAGCTGGCTGACTTT 1845
4r GGGATGTGCAAGGAAGGGATTCTGAATGGCGTGACAACTA.CACCTTCTGT 34
PKC ε GGGATGTGCAAGGAAGGGATTCTGAATGGCGTGACAACTACCACCTTCTGT 1896
593K
4r GGCACCCCGAACTACATCGCCCCCGAGATCCTG 1
PKC ε GGGACTCCTGACTACATAGCTCCAGAGATCCTG 1929

Figure 6.5 Identification of sequences encoding PKC ζ by restriction analysis.

Products of PCR amplification were ligated into the pGemT vector and transformed into *E coli.* An aliquot of the DNA prepared from each clone was analysed by digestion with the restriction endonucleases, HindIII and RsaI in the One-for-All restriction enzyme buffer (1.5x)(Promega). a) shows the location of restriction sites for these enzymes in a construct containing a PCR fragment encoding PKC ζ. Numbers refer to positions in the cloning vector, or in the amplified PCR product (in italics). b) shows the expected size of the fragments obtained from cleavage of a plasmid containing the sequence encoding PKC ζ. c) Agarose gel electrophoresis of typical restriction digests showing a PKC ζ containing plasmid (photo ii) and a plasmid containing a PCR product with a different sequence (photo i). Photo (i) shows the fragment corresponding to the linearised construct, while photo (ii) shows the two fragments obtained from plasmid with an insert encoding PKC ζ. Sizes of the DNA molecular weight markers are shown in base pairs.

Figure 6.5

a)



b)

Fragment	Expected size (base pairs)
Vector+Insert (RsaI-HindIII)	1421 (1232)
Vector+Insert (HindIII-RsaI)	1929 (2064)

c)

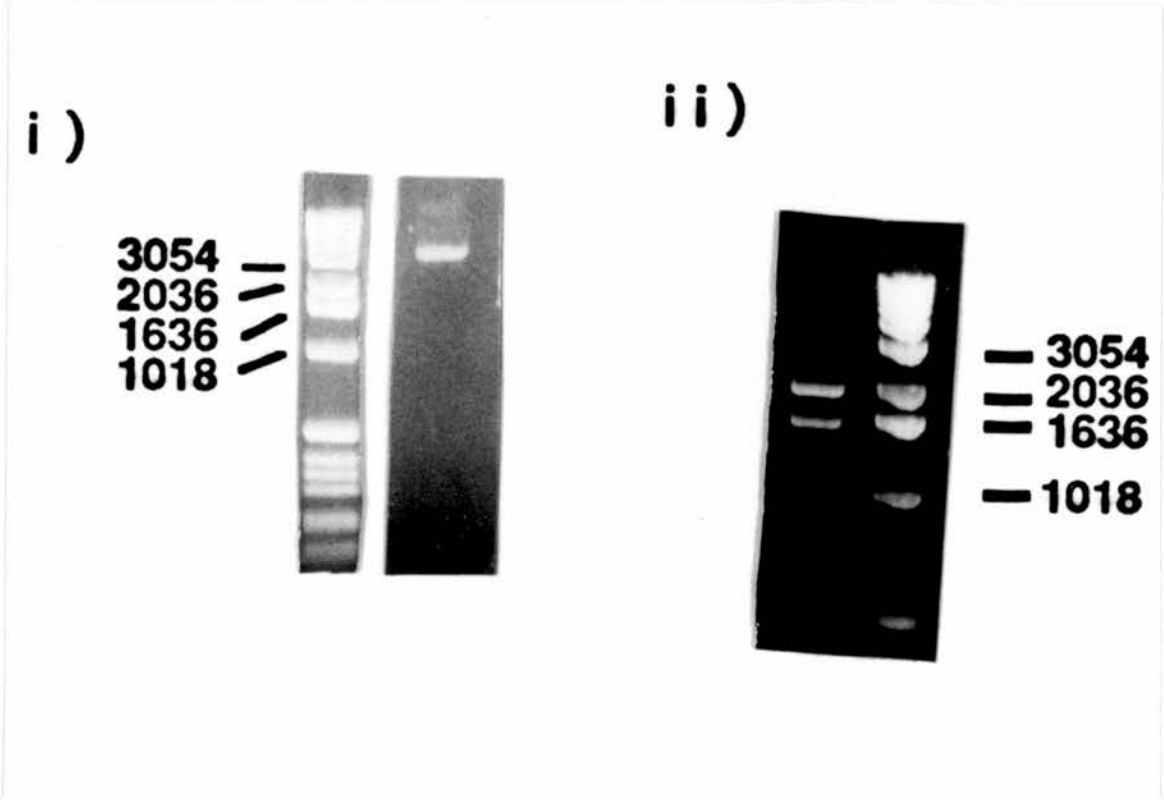


Figure 6.6 DNA sequencing analysis of a clone containing a PCR product encoding PKC ζ.

The identity of some of the plasmids indicated by restriction analysis as containing fragments encoding PKC ζ was confirmed by DNA sequencing analysis using the dideoxy method. The sequence of one such plasmid, 4n is shown aligned with the nucleotide sequence for rat PKC ζ as reported in the GenBank™ data base. Bars indicate identical nucleotides and the site of cleavage by HindIII, the endonuclease used in restriction analysis, is shown by the arrow. The identity of the each sequence is shown on the left while the nucleotide numbers are shown on the right. Sequences representing the PCR primers are indicated by the shaded boxes; ▨ 592K, ▧ 593K.

Figure 6.6

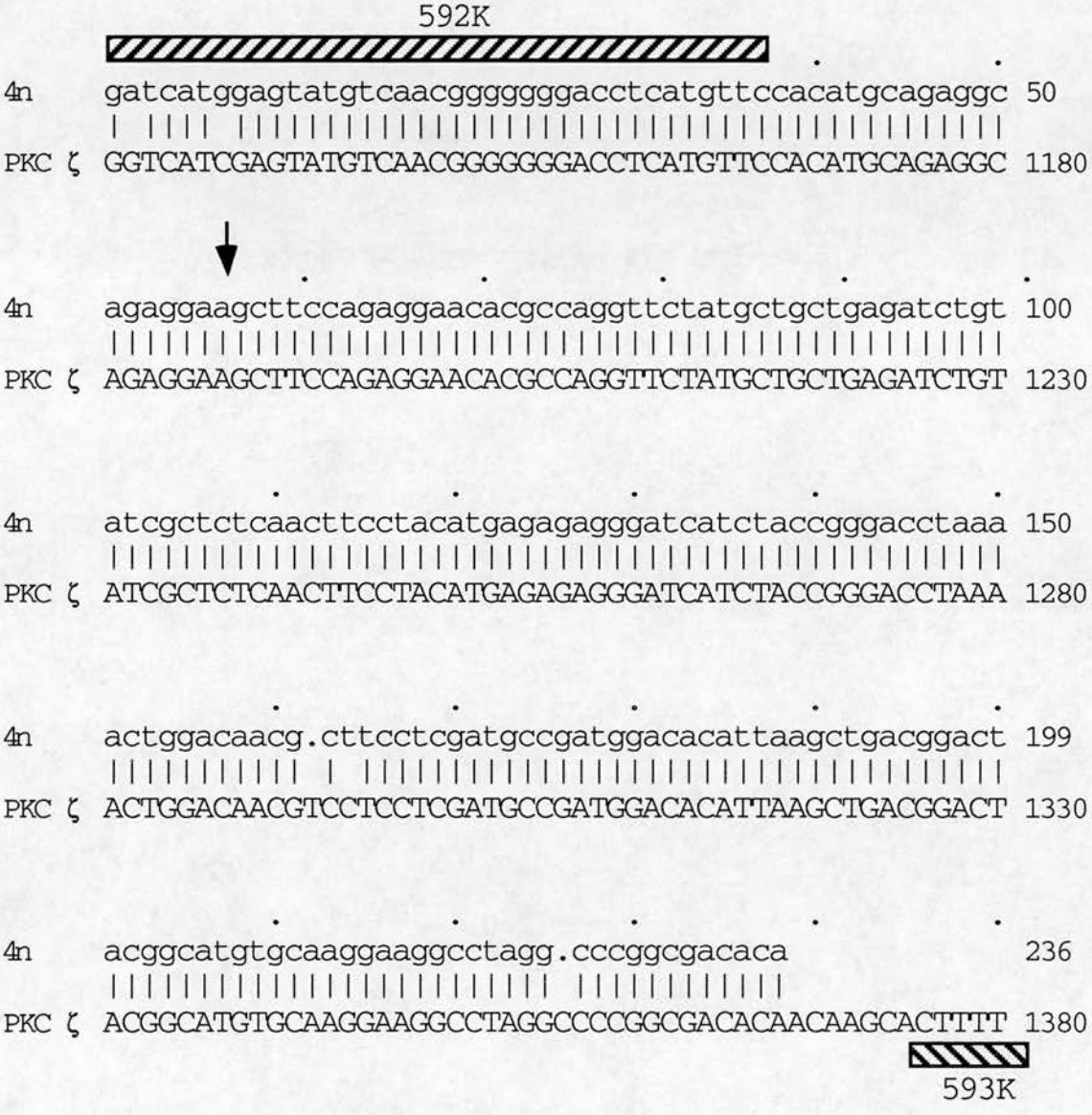

592K
4n gatcatggagtatgtcaacgggggggacctcatgttccacatgcagaggc 50
PKC ζ GGTCATCGAGTATGTCAACGGGGGGGACCTCATGTTCCACATGCAGAGGC 1180
4n agaggaagcttccagaggaacacgccaggttctatgctgctgagatctgt 100
PKC ζ AGAGGAAGCTTCCAGAGGAACACGCCAGGTTCTATGCTGCTGAGATCTGT 1230
4n atcgctctcaacttcctacatgagagagggatcatctaccgggacctaaa 150
PKC ζ ATCGCTCTCAACTTCCTACATGAGAGAGGGATCATCTACCGGGACCTAAA 1280
4n actggacaacg.cttcctcgatgccgatggacacattaagctgacggact 199
PKC ζ ACTGGACAACGTCCTCCTCGATGCCGATGGACACATTAAGCTGACGGACT 1330
4n acggcatgtgcaaggaaggcctagg.cccggcgacaca 236
PKC ζ ACGGCATGTGCAAGGAAGGCCTAGGCCCCGGCGACACAACAAGCACTTTT 1380
593K

Figure 6.7 DNA sequencing analysis of a clone containing a PCR product encoding PKC α.

Plasmids indicated by restriction analysis as containing fragments of the expected size but identified as encoding neither PKC ε or ζ were sequencing analysis using the dideoxy method. The sequence of one such plasmid, P1, which was found to encode PKC α is shown aligned with the nucleotide sequence for rat PKC α as reported in the GenBank™ data base. Bars indicate identical nucleotides and the sequence representing the PCR primers 593K is indicated by the shaded box ▨. The identity of the each sequence is shown on the left while the nucleotides are numbered on the right.

Figure 6.7

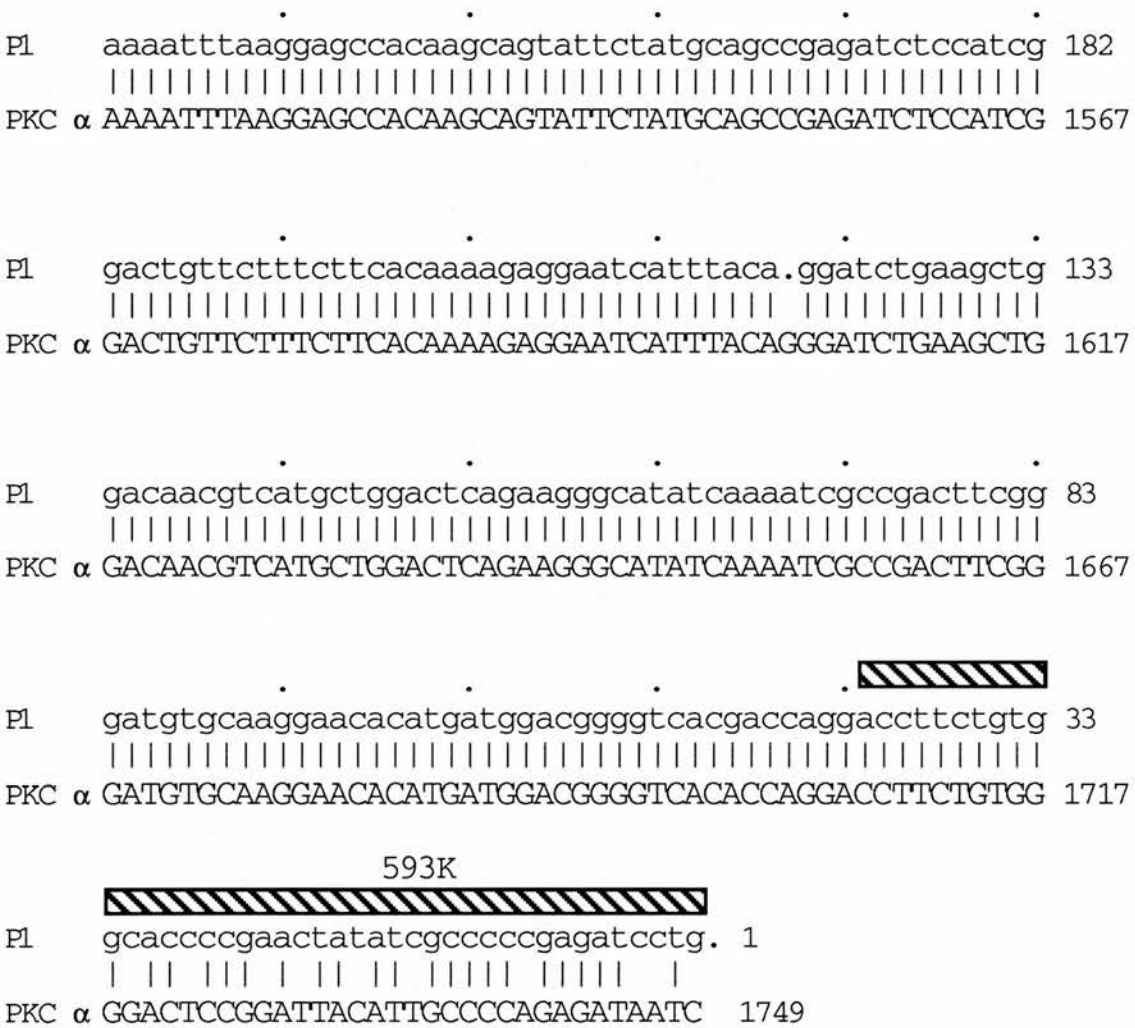

P1 aaaatttaaggagccacaagcagtattctatgcagccgagatctccatcg 182
PKC α AAAATTTAAGGAGCCACAAGCAGTATTCTATGCAGCCGAGATCTCCATCG 1567
P1 gactgttctttcttcacaaaagaggaatcatttaca.ggatctgaagctg 133
PKC α GACTGTTCTTTCTTCACAAAAGAGGAATCATTTACAGGGATCTGAAGCTG 1617
P1 gacaacgtcatgctggactcagaagggcatatcaaaatcgccgacttcgg 83
PKC α GACAACGTCATGCTGGACTCAGAAGGGCATATCAAAATCGCCGACTTCGG 1667
P1 gatgtgcaaggaacacatgatggacggggtcacgaccaggaccttctgtg 33
PKC α GATGTGCAAGGAACACATGATGGACGGGGTCACACCAGGACCTTCTGTGG 1717
593K
P1 gcaccccgaactatatcgcccccgagatcctg. 1
PKC α GGACTCCGGATTACATTGCCCCAGAGATAATC 1749

Figure 6.8 Agarose gel electrophoresis of products from PCR amplification of cDNA encoding PKC isoforms expressed in rat anterior pituitary gland.

10 μl of the reaction mixture after PCR amplification with PC3 and 593K was analysed by electrophoresis through a 1% agarose gel containing 0.5 mg/ml ethidium bromide. A strong band was visible at approximately 500 bp (as indicated by the arrow), the predicted size for PKC isoforms α-ι. Sizes of the molecular weight markers are indicated in base pairs.

Figure 6.8

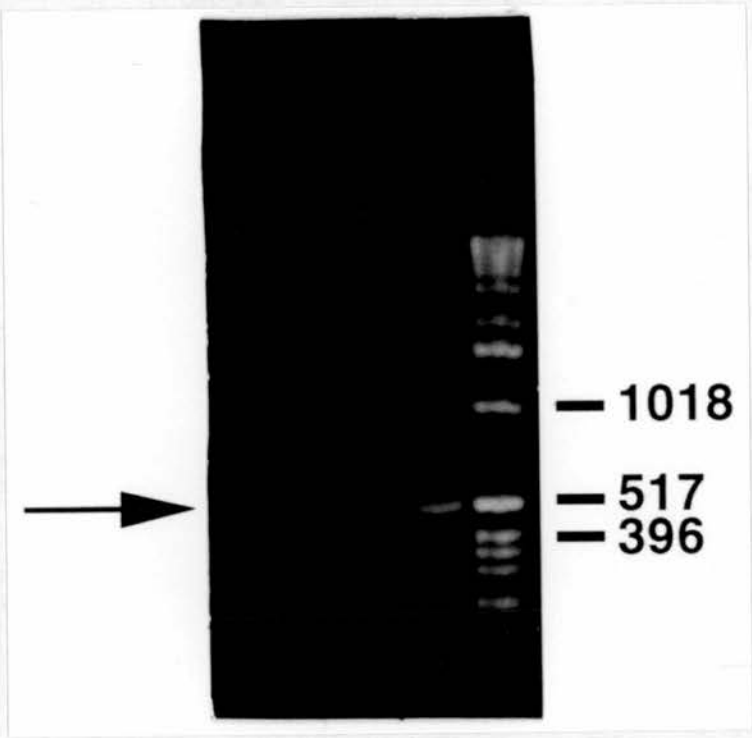
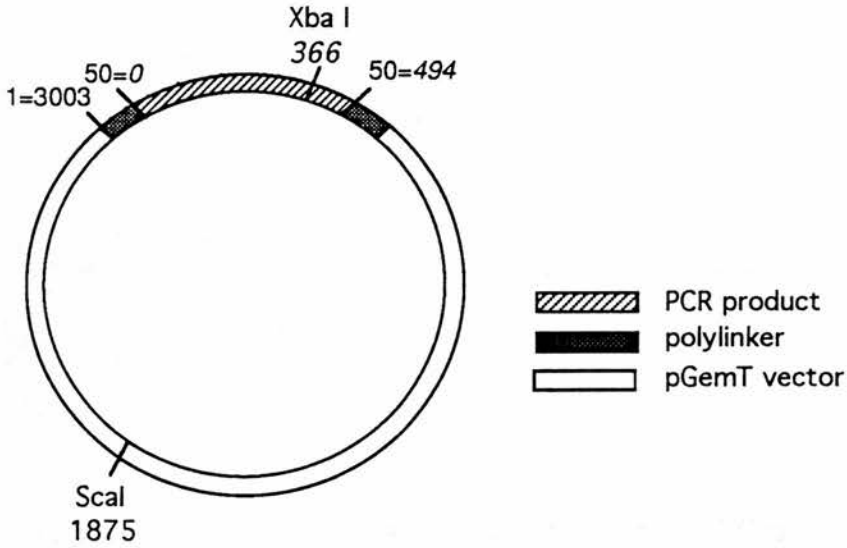


Figure 6.9 Identification of sequences encoding PKC ε by restriction analysis.

Products of PCR amplification using PC3 and 593K as primers were cloned and an aliquot of the DNA prepared from each clone was analysed by digestion with the restriction endonucleases, XbaI and SacI in the One-for-All restriction enzyme buffer (1.5x)(Promega). a) shows the location of restriction sites for these enzymes in a construct containing a clone encoding PKC ε. Numbers refer to positions in the cloning vector, or in the amplified PCR product (in italics). b) shows the expected size of the fragments obtained from cleavage of a clone containing the sequence encoding PKC ε. c) Agarose gel electrophoresis of typical restriction digests showing a clone of a PCR product of unknown sequence (i) and a PKC ε clone (ii). Sizes of the DNA molecular weight markers are shown in base pairs.

Figure 6.9

a)



b)

Fragment	Expected size (base pairs)
Vector+Insert (ScaI-XbaI)	1544 (1306)
Vector+Insert (XbaI-ScaI)	1953 (2191)

c)

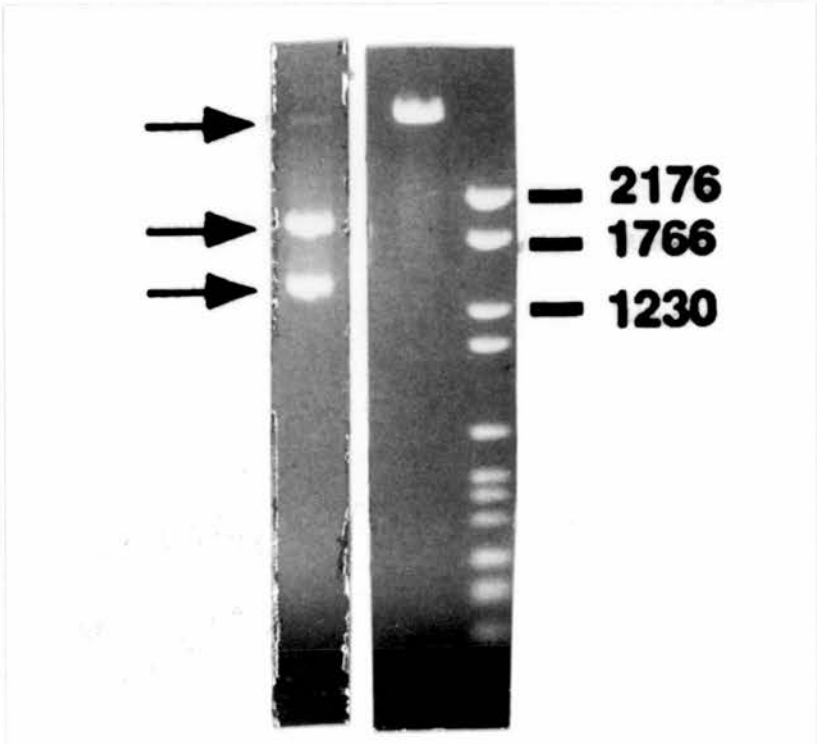
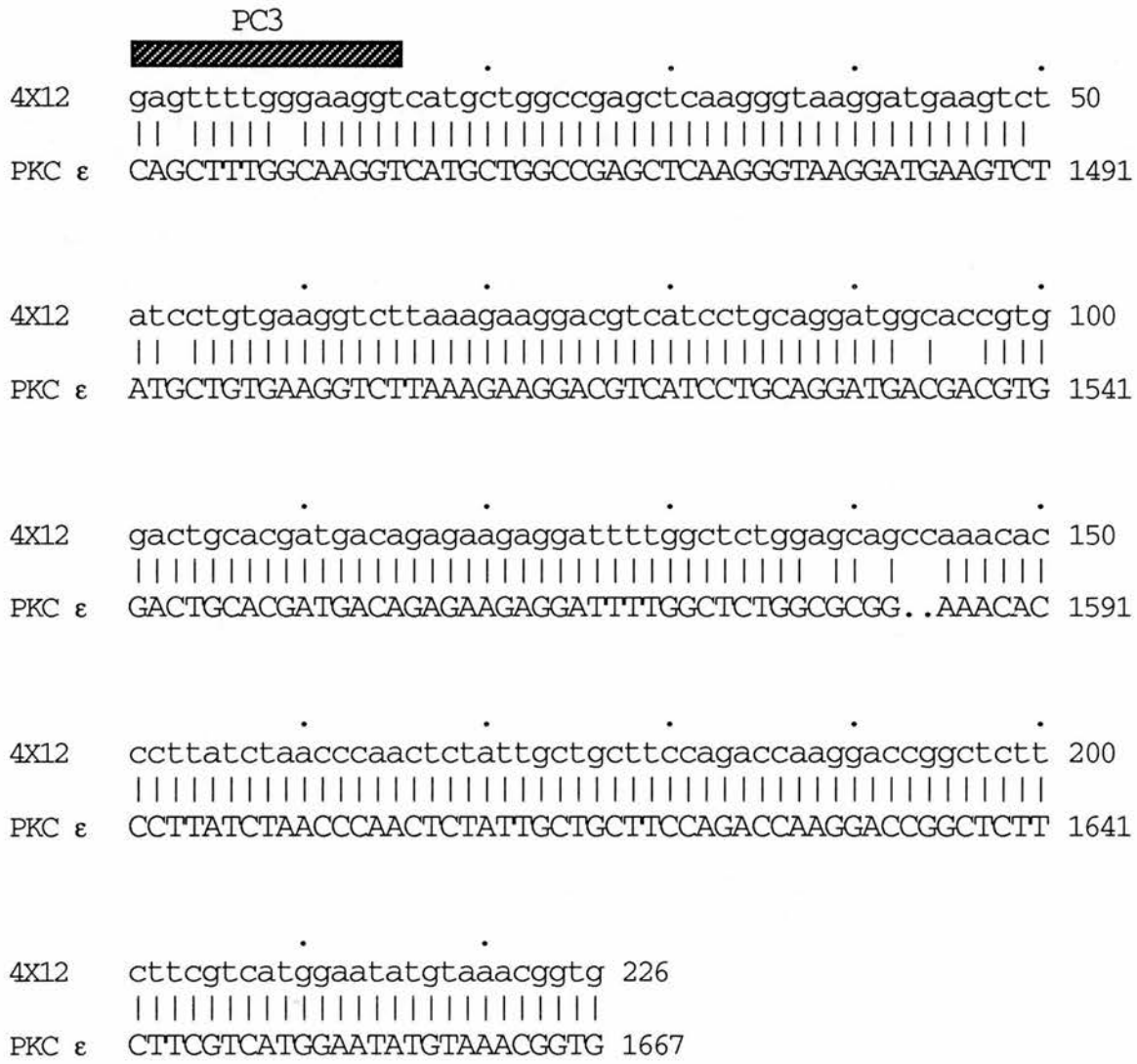


Figure 6.10 DNA sequencing analysis of a clone containing a PCR product encoding PKC ε.

The identity of some of the clones indicated by restriction analysis as containing fragments encoding PKC ε was confirmed by DNA sequencing analysis using the dideoxy method. The sequence of one such clone, 4X12 is shown aligned with the nucleotide sequence for rat PKC ε as reported in the GenBank™ data base. The identity of the each sequence is shown on the left while the nucleotides are numbered on the right. The sequence representing the PCR primer PC3 is indicated by the shaded box  .

Figure 6.10


PC3
4X12 gagttttgggaaggtcatgctggccgagctcaagggtaaggatgaagtct 50
PKC ε CAGCTTTGGCAAGGTCATGCTGGCCGAGCTCAAGGGTAAGGATGAAGTCT 1491
4X12 atcctgtgaaggtcttaaagaaggacgtcatcctgcaggatggcaccgtg 100
PKC ε ATGCTGTGAAGGTCTTAAAGAAGGACGTCATCCTGCAGGATGACGACGTG 1541
4X12 gactgcacgatgacagagaagaggattttggctctggagcagccaaacac 150
PKC ε GACTGCACGATGACAGAGAAGAGGATTTTGGCTCTGGCGCGG..AAACAC 1591
4X12 ccttatctaacccaactctattgctgcttccagaccaaggaccggctctt 200
PKC ε CCTTATCTAACCCAACTCTATTGCTGCTTCCAGACCAAGGACCGGCTCTT 1641
4X12 cttcgtcatggaatatgtaaacggtg 226
PKC ε CTTCGTCATGGAATATGTAAACGGTG 1667

CHAPTER 7

OVERVIEW

This present study has shown that the H7-resistant form of PKC which is involved in a number of anterior pituitary cell responses (MacEwan et al., 1992; Thomson et al., 1993b) can be detected *in vitro* in a mixed micelle thiophosphorylation assay using extracts of cytosol from anterior pituitary. As illustrated in Chapter 3, this H7-resistant PKC activity is PS-dependent but Ca^{2+}-independent and is stimulated by the tumour promoters PDBu and mezerein and also by the synthetic diglyceride DOG. H7-resistant PKC activity is detected when histone IIIS and glycogen synthase peptide are the substrate for thiophosphorylation but not with the α or ε pseudosubstrate peptides as the substrate, as these peptides are particularly efficiently phosphorylated by PKC ε (Koide et al., 1992), one of the predominant nPKCs present in anterior pituitary cytosol. H7-resistant PKC-like kinase activity was present in anterior pituitary tissue and to a lesser extent lung but not in midbrain, spleen, COS 7 cells or a range of other tissues tested. This tissue distribution is distinct from that of the well characterised PKCs α-ζ (Wetsel et al., 1992), the majority of which have been reported to be sensitive to H7 (Pelosin et al., 1990). Further purification by HAP chromatography showed that anterior pituitary is unusual in containing PKC activity which elutes after the major peak of PKC α immunoreactivity, in a position where little activity is detected on HAP separation of PKCs from the majority of the other tissues investigated. Activity was also found to elute in this position on HAP fractionation of extracts from lung and from the pituitary-derived αT3-1 cell line, both thought to contain the H7 resistant PKC (Chapter 3)(Fennell et al., 1994; Sim and Mitchell, 1994).

Pharmacological analysis of the enzymatic activity in HAP fractions from pituitary, αT3-1 cells and lung showed that the PKC eluting in this position, but not in earlier fractions, was relatively resistant to H7 . There is

only one previous report of activity eluting from HAP columns in this position, in an extract from rat retina (Fujisawa et al., 1992). This PKC activity has yet to be characterised but our analysis of PKC activity partially purified from rat retina found no evidence of H7-resistant activity in this tissue.

Immunoblotting of pituitary HAP fractions showed that only immunoreactivity for PKC α and ζ was detected in the HAP fractions containing H7-resistant activity. Protein kinase C α is dependent on Ca^{2+} for activation (Nishizuka, 1992) and has been reported to be sensitive to H7 (Pelosin et al., 1990). Protein kinase C ζ activity *in vitro* has been shown to be independent of phorbol ester stimulation (Gschwendt et al., 1992; Nakanishi and Exton, 1992) and this isoform, unlike the H7-resistant PKC is reported to be less sensitive to inhibition by staurosporine and other related bisindolylmaleimides (Kochs et al., 1993b, Martiny-Baron et al., 1993; McGlynn et al., 1992). Immunoreactivity for PKC ζ is visible as a doublet of 79 and 88 kDa in pituitary HAP fractions. There are reports to suggest that the high molecular weight band of these may be a result of cross reaction with another PKC isoform, possibly PKC α, (Batlle et al., 1994; Tsutumi et al., 1993) as this band is translocated and downregulated in response to phorbol esters. In order to investigate the possibility of an extended form of PKC ζ that is responsive to phorbol esters being present in anterior pituitary cells, PCR was conducted using zeta-specific primers (Chapter 5) but this approach found no evidence for such a hypothetical PKC ζ-related kinase in anterior pituitary or αT3-1 cells.

To determine the possible identity of the H7-insensitive kinase, HAP fractions were allowed to autophosphorylate and subsequent SDS-PAGE revealed a 140 kDa protein present only in the fractions containing H7-resistant PKC activity. A corresponding 130 kDa protein was detectable in

the same fractions after immunoblotting with an anti-PKC antibody raised to a consensus sequence conserved between PKC isoforms. Both the 140 kDa autophosphorylation signal and the corresponding PKC-like immunoreactivity were also present in the H7-resistant HAP fractions from lung (Chapter 4 and (McCulloch et al., 1994)) but not in the corresponding HAP fractions from midbrain, a tissue which does not contain H7-resistant PKC activity. Therefore the elution profile from HAP chromatography and the tissue distribution of this high molecular mass autophosphorylation signal correlates with the H7-resistant PKC activity, and the reactivity with the PKC consensus antibody suggests it may be the H7-resistant PKC itself rather than a co-eluting substrate protein. This is further supported by preliminary studies conducted in this laboratory which have shown that the H7-resistant PKC activity can be separated from some of the other pituitary PKCs using 100 kDa molecular mass cut-off filters, and by subsequent gel filtration studies which detected H7-resistant PKC activity in fractions corresponding to a molecular mass of 120-150 kDa (D McCulloch and R Mitchell; unpublished data).

Thus the H7-resistant PKC appears to correspond to a protein with a molecular mass of approximately 130 kDa. This size is larger than that of the well characterised PKC isoforms (PKC α-ι), which vary between 65-90 kDa (Nishizuka, 1992), and this protein is not detected by isoform-specific antisera for PKC α, β, δ, θ, ε and ζ (Chapter 4). While the lack of immunoreactivity for these isoforms does not rule out the possibility of the H7-resistant kinase being a modified form of one of these isoforms (for example a phosphorylated form), as it is conceivable that such modifications could disrupt antigenicity, the particularly high molecular mass makes this possibility unlikely. This is not the only report of a PKC with a higher molecular mass than 90 kDa. Recently, immunoreactivity for

high molecular mass forms of PKC η (97 kDa)(Sublette et al., 1993a) and PKC ζ (160 kDa)(Sacktor et al., 1993) has been detected in the CA1 region of the hippocampus and these forms have been proposed to be involved in the maintenance of long term potentiation (Sublette et al., 1993b). A 95 kDa PKC which is detected using an anti-PKC η antibody has also been detected in platelets and is involved in the signal transduction stimulated by platelet activating factor (Wang et al., 1993). Furthermore a novel membrane bound isoform, PKC μ that has a molecular mass of 117 kDa has recently been cloned. Thus on the basis of its high molecular mass of 130 kDa, distinct elution profile from HAP chromatography, tissue distribution and characteristic resistance to H7 but not other PKC inhibitors it seems likely that this kinase represents a novel isoform of PKC.

Future Directions

To identify and further characterise this novel form of PKC, it will be necessary to obtain a partial sequence which can be used to clone it. There are two obvious approaches which may be used to obtain this:

Homology cloning could be conducted using degenerate PCR primers to consensus PKC sequences as this approach has been successfully used to clone and identify PKC θ, ι and μ . Initial attempts at this approach as outlined in Chapter 6 succeeded in cloning PCR fragments encoding PKC ε, ζ and α but no novel PKC related sequences were obtained. If the H7-resistant PKC is a novel isoform, it should be possible to amplify a fragment by PCR, provided it is homologous to the other PKC isoforms in the regions to which the PCR primers are designed. It would therefore be necessary to design alternative primers to those used in Chapter 6, especially since those would have been unable to amplify PKC μ if present and so potentially other PKC-related kinases may also have

remained undetected. A rapid method, for example colony hybridisation, would also be needed for analysis of the resulting clones containing PCR fragments, as the majority of such clones are likely to encode the PKC isoforms most abundantly expressed in anterior pituitary. This would enable further analysis to be conducted only on those colonies containing the less abundant PKCs.

An alternative approach for obtaining a partial sequence is by protein purification. The extensive purification procedures that have been used previously for isolating PKC isoforms would not be viable for the H7-resistant PKC as they require large quantities of protein. However, as this PKC can be recognised by its unique autophosphorylation signal, 2D SDS PAGE could be used to purify this protein. Preliminary experiments using this method have identified the H7-resistant PKC on 2D gels following autophosphorylation of the relevant HAP fraction using ^{32}P labelled ATP (J Simpson and R Mitchell; unpublished results). By this method the kinase could be separated from the other proteins present in the same HAP fraction and extracted for microsequencing. This would however require more protein than is currently obtained from HAP fractionation of 50 male rat pituitaries. It may therefore be necessary to obtain the H7-resistant kinase from an alternative tissue source. Potential candidates are rat lung, αT3-1 cells (which may be grown in large quantities) or sheep pituitary, as this species was also found to contain this form of PKC (Chapter 3). This method would result in a short amino acid sequence from the H7-resistant PKC which could then be used to design degenerate primers for PCR in conjunction with a PKC consensus sequence, in order to generate a larger fragment.

Both these possible strategies would result in the generation of a portion of the sequence encoding the H7-resistant kinase. At this stage

there are once again two possible approaches which may be adopted to obtain the full length clone of this PKC. The fragment obtained could be radiolabelled and used as a probe for screening a pituitary cDNA library under conditions of high stringency to obtain clones which may be sequenced to reveal the primary structure of this protein. Alternatively, an anchored PCR procedure, RACE (rapid amplification of cDNA ends) could be employed. This approach uses gene specific primers and either the natural poly (A) tail of the mRNA (3'-RACE) or a poly (A) tail added to specific first strand cDNA ends by terminal deoxynucleotidyl transferase as PCR priming sites to result in amplification of fragment encoding the full length open reading frame.

Cloning and sequencing of the H7-resistant PKC would enable further investigation into the role of this kinase in cellular signalling pathways. Its distribution could be determined by Northern and in situ analysis, as, although it appears to be predominantly expressed in anterior pituitary, it may be present at lower levels in other tissues. Furthermore its subcellular distribution is as yet unknown. The consequences of overexpression of this isoform in host cells and its deletion in normal cells by either the expression of dominant negative mutants or the use of antisense oligonucleotides could provide further information on the role of this kinase in cellular responses.

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BIOCHEMICAL CHARACTERISTICS OF AN H7-RESISTANT FORM OF PROTEIN KINASE C FROM ANTERIOR PITUITARY TISSUE

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Abbreviations used: PKC, Protein kinase C; PDBu, phorbol 12, 13-dibutyrate; H7, 1-(5-isoquinoline sulphonyl)-2-methyl-piperazine hydrochloride; HAP, hydroxyapatite

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ABSTRACT

A number of cellular responses mediated by protein kinase C (PKC) in the anterior pituitary gland involve a form of PKC that is unusually resistant to the PKC inhibitor H7. We have previously shown that a component of the Ca^{2+}-independent phorbol 12, 13-dibutyrate (PDBu)-induced PKC enzymic activity from this tissue has a corresponding resistance to H7 and that the PKC species responsible is detected predominantly in the anterior pituitary gland. The present study showed initially that DEAE-cellulose chromatography can separate Ca^{2+}-independent PKC activity from anterior pituitary tissue into fractions with different sensitivities to H7. Furthermore fractionation by hydroxyapatite (HAP) chromatography of both anterior pituitary and midbrain extracts resulted in three main peaks of PKC activity with only anterior pituitary containing significant PDBu-induced PKC activity which eluted after the third main peak. Ca^{2+}-independent activity in this fraction was sensitive to the PKC inhibitor Ro 31-8220 but relatively resistant to H7. Immunoblotting with antisera specific for PKC α, β_1, δ, ε, ζ and θ showed that this fraction did not contain the major immunoreactivity for any of these isoforms. Phosphatidylserine-dependent autophosphorylation of HAP fractions from anterior pituitary revealed the presence of a 140 kDa protein which was not seen in midbrain and whose distribution between the fractions correlated with the H7-resistant PKC activity. Similarly, immunoblotting with an antiserum raised to a PKC consensus sequence (in the catalytic domain) showed a prominent immunoreactive species at approximately 130 kDa which was present solely in those anterior pituitary HAP fractions displaying H7-resistant activity. Size fractionation using 100 kDa cut-off filters showed that, in cytosol from anterior pituitary but not midbrain, there was H7-resistant PDBu-induced activity in the fraction containing proteins of molecular mass greater than 100 kDa. This 130 kDa protein may well represent the H7-resistant kinase and may be related to one of the known PKCs or a novel isoform of PKC.

INTRODUCTION

The protein kinase C (PKC) family of phospholipid-dependent serine/threonine kinases has been implicated in a wide variety of cellular processes, including growth, differentiation, control of gene expression and secretion of hormones and neurotransmitters [1, 2, 3]. There are now known to be at least eleven different isoforms, the product of ten genes, each isoform varying in its structure and biochemical properties [4]. They may be divided into subgroups on the basis of their activation characteristics. The classical cPKCs (α, β_1, β_2 and γ) are dependent on Ca^{2+} for activation while the novel nPKCs (δ, ε, η and θ) and atypical aPKCs (ζ, ι and μ) are all Ca^{2+}-independent and lack the proposed Ca^{2+} binding region, the C2 domain [5]. The cPKCs and nPKCs are activated by diglycerides and the tumour-promoting phorbol esters, but the aPKCs have modifications in the C1 domain containing the cysteine-rich regions thought to compose the diglyceride/phorbol ester binding site [6, 7, 8] and are reported to be unaffected by these compounds [9, 10]. The eleven isoforms have been shown to differ widely in their substrate specificity [11] and phospholipid dependence [12] as well as in their tissue and cellular localisation [13, 14].

A number of studies have found that PKC activators and inhibitors vary in their ability to influence different PKC-mediated responses, possibly due to the differential sensitivity of certain isoforms to these pharmacological agents [15, 16, 17, 18]. We have previously shown in the anterior pituitary that a number of physiological processes are mediated by a form of PKC which, although sensitive to the PKC inhibitors staurosporine and Ro 31-8220, is unusually resistant to another PKC inhibitor H7 [19, 20, 21, 22]. This kinase, detectable in anterior pituitary cytosol using a mixed-micelle PKC activity assay, is activated by phorbol esters in the absence of Ca^{2+} but does not appear to correspond to any of the well-characterised PKC isoforms [23]. In this study we show that this H7-resistant PKC can be biochemically separated from other pituitary PKCs by

DEAE cellulose and hydroxyapatite (HAP) chromatography, and present evidence to suggest that it may represent a novel form of PKC.

EXPERIMENTAL

DEAE cellulose separation

Anterior pituitaries from 20 male Wistar rats were homogenised in 2 volumes of 20 mM Tris HCl (pH 7.5) containing 50 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF; Sigma Chemical Company, Poole, Dorset, UK), 0.01% (w/v) leupeptin (Sigma) and 20 μM trans-epoxysuccinyl-l-leucylamido (4-guanidino) butane (E64; Sigma), using a Ystral polytron homogenizer (Scientific International Industries Ltd, Loughborough, Leics, UK). The homogenate was centrifuged (16 000 ***g***, 20 min, 4°C); the supernatant was collected and recentrifuged for 5 min. The second supernatant was taken to represent cytosol and the PKCs present were partially purified on DEAE cellulose (DE52; Whatman International Ltd, Maidstone, Kent, UK)(0.5 ml) in a Bio-Rad Poly-Prep chromatography column (Bio-Rad Laboratories, Richmond, California, USA) at 4°C. After sample loading, the matrix was then washed with 6 column volumes of homogenisation buffer before the partially-purified PKC was eluted with 3 column volumes of buffers containing 50, 100, 150 and 200 mM NaCl sequentially. The PKC activity in each eluate was then assessed.

Purification by hydroxyapatite chromatography

PKC isoforms were resolved by HAP chromatography (Biogel HT, Bio-Rad Laboratories, Watford, Herts, UK) using a procedure based on that published in [24, 25] and similar to that described in [26] . Midbrain or anterior pituitary tissue from male Wistar rats was homogenised in 6 volumes of ice-cold buffer (25 mM Tris/HCl, 250 mM sucrose, 2 mM EDTA, 10 mM EGTA, 50 mM 2-mercaptoethanol) containing 1 mM PMSF, 1 mM benzamidine, 2 μg/ml soya bean trypsin inhibitor and 25 μg/ml leupeptin and centrifuged (10 min, 6000 ***g***,

4°C). The resulting supernatant was strained through glass wool and Triton X-100 was added (final concentration 1% (v/v)) before further centrifugation (60 min, 105 000 ***g***, 4°C). The PKCs in the supernatant were partially purified on DEAE cellulose columns as described above but the elution buffer contained 300 mM NaCl. This fraction was dialysed into 5 mM potassium phosphate buffer (pH 7.5) containing 10% (v/v) glycerol, 0.5 mM EDTA, 0.5 mM EGTA, 1 mM dithiothreitol and loaded onto a HAP column equilibrated in the same buffer, which was also used to wash the column (4 volumes). Proteins were eluted in a linear potassium phosphate gradient (5-300 mM; fraction volume 2.5 ml) and the PKC activity was assayed. Fractions were pooled as appropriate, and dialysed against 50% glycerol, 10 mM Tris HCl, 0.5 mM EDTA, 0.5 mM EGTA, 25 mM 2-mercaptoethanol (pH 7.5) for storage at -20 °C.

Mixed micelle PKC activity assay

The PKC activity partially-purified from cytosol or in the more extensively purified HAP eluate was determined as the phorbol 12, 13-dibutyrate (PDBu)-induced thiophosphorylation of GS peptide ([Pro, Leu, Ser, Arg, Thr, Leu, Ser, Val, Ala, Ala, Lys, Lys], modified from the sequence of residues 1-12 of glycogen synthase [27]), measured in the presence of phosphatidylserine (PS). All of the activity measured was PS-dependent as substitution with phosphatidylcholine, which is unable to act as a cofactor in PKC activation [28], abolished it. A mixed-micelle assay was used in this study to enable the Ca^{2+}-dependent and -independent activity to be analysed separately, as PKC activation is obligatorily dependent on phorbol/diglyceride activators under these conditions [29]. The methods used were modified from those in references [30, 31] and are similar to those described in [23]. Protein kinase C activity was measured in an assay mixture (total volume 50 µl) containing, unless otherwise stated, (final concentrations): 10 mM $MgCl_2$, 200 µg/ml PS (sodium salt) (Lipid Products, Nutfield, Surrey, UK), 0.04% Nonidet P-40 (Calbiochem, Novabiochem, Nottingham, UK), 200 µM GS peptide (Bachem Ltd, Saffron Walden, Essex, UK), 50 µM ATP-γ-[^{35}S] (NEN, DuPont, Dreiech, Germany)(0.6 µCi/tube), 1µM PDBu

(LC Laboratories, Calbiochem) and 10 µl cytosol or HAP eluate. Assay tubes also contained either 600 µM $CaCl_2$ (100 µM free Ca^{2+}) or 5 mM EGTA (less than 3 nM free Ca^{2+}) and inhibitors at various concentrations. (H7, Ro 31-8220 and GF109203X were from LC Laboratories, Roche Products Ltd, Welwyn, Herts, UK and Calbiochem respectively). All assay components and drugs were dissolved in 20 mM Tris HCl (pH 7.5) with 0.5 mM EGTA. Reactions were started by the addition of enzyme, incubated at 30°C for 15 min and stopped by addition of 20 µl TCA (6.8% w/v final). After incubation on ice for 15 min, TCA-precipitable material was removed by centrifugation and the supernatant was spotted onto 2x2 cm pieces of P-81 cellulose phosphate ion-exchange chromatography paper (Whatman International Ltd, Maidstone, Kent, UK), which were washed extensively in 75 mM H_3PO_4, dried and counted by liquid scintillation.

Data Analysis

Basal activity with PS alone (at appropriate inhibitor concentrations) was subtracted from the inhibitor curves in the absence/presence of Ca^{2+}. A normalised asymmetric sigmoid Hill curve was fitted to the Ca^{2+}-independent PDBu-induced activity and subtracted from the evoked activity values obtained in the presence of Ca^{2+}. A normalised curve was then similarly fitted to the resulting values for Ca^{2+}-dependent activity. Curve fitting was carried out using the iterative error-weighted curve fitting program, P.fit (Elsevier Biosoft, Cambridge, UK).

Immunoblotting with antibodies to PKC isoforms

Pituitary HAP fractions were diluted 1:1 (v/v) with 50 mM Tris/HCl (pH 7.2), or, for immunostaining of PKC α, β_1, ζ and θ, concentrated 5 fold and resuspended in the same buffer using a Centricon-30 ultrafiltration device (Amicon, Stonehouse, Gloucs, UK) then heated at 100°C for 5 min in the presence of 2% (w/v) SDS and 5% (v/v) 2-mercaptoethanol. Four µl or, for immunostaining of PKC δ, 1 µl aliquots were applied to 7.5% homogenous microgels and SDS-PAGE and electroblotting were performed using a

PhastSystem apparatus (Pharmacia Biotech, Milton Keynes, Bucks, UK). PKC isoforms were identified with rabbit polyclonal antisera raised to isoform-specific peptide sequences in α, δ, ε and ζ (2 μg/ml)(Gibco BRL, Paisley, Renfrew, UK) and β_1 (2.5 fold dilution [32]. The specificity of staining with these antisera was confirmed in each case by use of antibody blocked by preincubation with the relevant antigenic peptide (1 μg/ml). PKC θ was detected using a mouse monoclonal antibody (1 μg/ml; Transduction Labs, Lexington, KY, USA). Both the midbrain and pituitary HAP fractions were also immunoblotted with a rabbit polyclonal consensus antibody raised to a sequence in the C4 domain conserved between all the PKC isoforms (PKC α:[Ac 543-550-Cys]) (Calbiochem). In each case the antibody reaction was visualised with horseradish peroxidase-labelled anti-IgG (Scottish Antibody Production Unit, Carluke, Lanarks, UK) followed by an enhanced chemiluminescence detection system (ECL; Amersham, Aylesbury, Bucks, UK).

Autophosphorylation of hydroxyapatite fractions

Midbrain and pituitary HAP fractions were incubated at 30°C in a mixture containing 13.3 mM Mops/KOH, 10.7 mM magnesium acetate, 66.7 μM [γ-^{32}P] ATP (approximately 10^6 cpm), 6.7 mM dithiothreitol, 4.2 mM 2-mercaptoethanol, 83 μM EDTA, 1 mM EGTA, 1 mM $CaCl_2$, 50 μg/ml PS, 3.3 mM Tris/HCl, 2 μg of BSA and 1 μg/ml protein kinase A peptide inhibitor [26]. After 30 min, reaction mixtures were disaggregated for SDS/PAGE and electrophoresed on 15 cm homogenous 8% gels. Phosphoproteins were located by autoradiography after gels had been fixed, stained with Coomassie Blue and destained.

Size fractionation of cytosolic PKC activity

Cytosol from midbrain and pituitary was prepared by DEAE cellulose separation as described above then diluted 12-fold in the homogenisation buffer before application to Centriprep-100 Filters (Amicon). After centrifugation twice (500 ***g***, 4°C, 15 min), the filtrate and similarly diluted retentate were reconcentrated by applying to DEAE cellulose columns as before.

RESULTS

Protein kinase C activity of pituitary DEAE-cellulose eluates

To investigate the possibility of biochemically separating the H7-resistant PKC from other nPKCs, anterior pituitary cytosol was fractionated on a DEAE-cellulose column, using sequentially buffers containing 50 mM, 100 mM, 150 mM and 200 mM NaCl. The Ca^{2+}-independent PDBu-induced PKC activity in each fraction was measured and the majority (50%) was detected in the 50-100 mM fraction. The 0-50 and 100-150 mM fractions contained 33% and 12% of the total Ca^{2+}-independent PKC activity respectively while the 150-200 mM fraction contained only 5% of the activity. The effect of H7 on the three fractions containing the most PDBu-induced activity was assayed and the IC_{50} values determined. H7-resistant activity was detected only in the 100-150 mM NaCl fraction (IC_{50} 77 $\pm$ 11 μM); PKC activity in the other fractions was sensitive to this inhibitor (Table 1).

Hydroxyapatite fractionation of PKC from anterior pituitary and midbrain

Figure 1 shows typical elution profiles of PKC activity from midbrain and anterior pituitary when fractionated by HAP chromatography. Midbrain was selected as a control tissue for comparison with anterior pituitary as midbrain contains all of the well-characterised PKC isoforms [33] but does not contain significant levels of the H7-resistant PKC [23] . Fractionation of midbrain extracts by HAP chromatography resulted in three main peaks of PKC activity as previously described for brain tissue [24], (Figure 1a). A substantial proportion of the activity in the smallest, initial peak was phospholipid-independent and may represent cleaved catalytic subunits of PKC. The other two peaks of PKC activity were both of similar height, though the first of these two eluted over more fractions than the second.

Hydroxyapatite chromatography of anterior pituitary extracts also revealed two main peaks of PS-dependent PKC activity (Figure 1b) although, in this case, the first one was much greater in magnitude than the second. However, unlike in midbrain, there was a small peak of activity after the second main peak (fraction

V) that was not seen in HAP profiles from other tissues examined, for example spleen, cerebellum, mammary gland and the COS 7 fibroblast cell line [26]. Samples were pooled and five fractions were collected from both pituitary and midbrain for further investigation, as shown in Figure 1.

The effect of H7 was examined on PDBu-induced PKC activity in pituitary HAP fractions I-V and fraction V was found to contain Ca^{2+}-independent activity that was relatively insensitive to this inhibitor (IC_{50} 85 ± 10 μM; Figure 2). Ca^{2+}-independent activity in fractions I-III was sensitive to H7 (IC_{50} values in the range 9-18 μM) while an intermediate IC_{50} value (45 ± 19 μM) was obtained for fraction IV (Table 2). Ca^{2+}-dependent activity was not detected in fraction II but was present in all other fractions and in each case it was H7-sensitive (IC_{50} values in the range 10-23 μM). All PDBu-induced activity was sensitive to inhibition by the selective PKC inhibitor Ro 31-8220 with IC_{50} values for all the Ca^{2+}-independent activity in fractions I-V in the range 103-169 nM. The Ca^{2+}-independent PKC activity in the corresponding fractions from midbrain was also examined and the effect of H7 determined. As seen in Table 3, all fractions were sensitive to this inhibitor with IC_{50} values varying between 17 and 35 μM.

Immunoblotting of anterior pituitary hydroxyapatite fractions with isoform-specific antibodies

Pituitary HAP fractions I-V were immunoblotted using antisera for specific PKC isoforms to allow comparison of the distribution of the H7-resistant activity with the elution pattern for each isoform. Figure 3 shows immunoblots for PKCs α, β_1, δ, ε, ζ, and θ, the isoforms reported to be present in anterior pituitary tissue ([34] and D J MacEwan (unpublished data)). Protein kinase C α, β_1, δ, ε and θ were all seen as bands in the 80-94 kDa range. Protein kinase C α immunoreactivity was present in fractions III-V, while fraction I contained the major immunoreactivity for PKCs β_1, δ and ε. PKC ζ immunoreactivity, seen as bands of 81 and 88 kDa, was present in all fractions, the 81 kDa band being strongest in fraction I while the 88 kDa band was predominant in fraction III. The monoclonal anti-PKC θ antibody recognised a 80 kDa band in fraction III similar

to that obtained in a Jurkat cell positive control [35]. This band was also just visible in fraction IV while in fraction I there was immunoreactivity at approximately 56, 68 and 73 kDa in I, (results not shown) which may represent PKC breakdown products or other cross reacting proteins. Immunoblots were not performed for either PKC γ or PKC η as no immunoreactivity has been detected for these isoforms in anterior pituitary [36][MacEwan, D. J., Johnson, M. S., Ohno, S. and Mitchell, R.; unpublished work].

Autophosphorylation of hydroxyapatite fractions

Hydroxyapatite fractions from both midbrain and pituitary were allowed to autophosphorylate in the presence of PS and Ca^{2+}, with ATP-γ [^{32}P] and the resulting labelled proteins were visualised by autoradiography. In both cases, a number of proteins were labelled in fractions I-IV. Prominent bands included, in fraction I, one of 88 kDa and, in fractions III and IV, one of approximately 95 kDa. These may well represent autophosphorylation of PKC β_1 and α respectively. In fraction V from pituitary there was one predominant labelled protein which had a molecular mass (when phosphorylated) of approximately 140 kDa (Figure 4). This protein was also present in pituitary fraction IV but was not seen in any of the midbrain fractions.

Immunoblotting of hydroxyapatite fractions with PKC consensus antibody

Hydroxyapatite fractions from both pituitary and midbrain were immunoblotted with an antiserum raised to a sequence in the C4 domain conserved between all the PKC isoforms (PKC α:[Ac 543-550-Cys]). This was to test whether the phosphorylation signal at 140 kDa represented autophosphorylation of a PKC distinct from the known species recognised by our isoform-specific antisera or whether it might be due to phosphorylation of a PKC substrate that coelutes from HAP with the H7-resistant PKC. Although a number of other proteins were labelled by this antiserum in both anterior pituitary and midbrain HAP fractions, the strongest staining was of a protein of approximately 130 kDa in anterior pituitary fractions IV and V (Figure 5). This immunoreactivity was also present to a lesser degree in fraction III but was not seen in the other

pituitary HAP fractions or any of those from midbrain. This protein may represent the unphosphorylated form of the 140 kDa autophosphorylation product.

Fractionation of cytosolic PKC activity by molecular mass

As shown in Figure 6a, when the DEAE eluate of the cytosol from midbrain was passed through a 100 kDa centrifugal filter, Ca^{2+}-independent PDBu-induced PKC activity in both the retentate and filtrate was sensitive to H7, with IC_{50} values of 5.8 ± 2.8 μM and 9.0 ± 1.7 μM respectively. However, in cytosol from anterior pituitary, a component of the Ca^{2+}-independent PKC activity in the filter retentate was markedly resistant to H7 and computer analysis for the inhibition curve for this data showed that a 2 site rather that a 1 site model gave a better fit (IC_{50} values 3.4 ± 1.4 μM (40.7 % of binding sites) and 260 ± 89 μM (59.3 % of sites)). In contrast the PKCs in the corresponding filtrate were H7 sensitive (IC_{50} value 8.2 ± 1.6 μM)(Fig 6b). The sensitivity of the H7-resistant PKC to other PKC inhibitors was examined by assaying the retentate activity in the presence of 30 μM H7 (sufficient to almost completely block the H7-sensitive component of the activity; Figure 6b). The selective PKC inhibitors Ro 31-8220 and GF 109203X gave corresponding IC_{50} values of 285 and 170 nM respectively on this H7-resistant activity, providing further evidence of its identity as a PKC-like enzyme.

Discussion

In this study we have shown that PDBu-induced PKC activity from anterior pituitary tissue may be biochemically separated into fractions with different sensitivities to the PKC inhibitor H7. This is consistent with previous reports that some but not all PKC-mediated responses in anterior pituitary cells are unusually resistant to this inhibitor [19, 20], and that relatively H7-insensitive PKC activity may be detected in cytosolic extracts from anterior pituitary tissue [23]. Both preliminary experiments using DEAE-cellulose and more extensive studies using HAP chromatography showed that it was possible to separate the H7-resistant activity from other PKCs (Table 1 and 2). Hydroxyapatite fractionation of pituitary

tissue extracts showed that some PKC activity eluted after the main peak of PKC α, at concentrations of potassium phosphate where little PKC activity was detected in midbrain (fraction V)(Figure 1b) or a number of other tissues [26]. Characterisation of the PKC activity showed that the Ca^{2+}-independent activity in this fraction was resistant to H7. One previous report has also described activity eluting in this position from rat retinal extracts [37]. However we have found no evidence of H7-resistant PKC activity in DEAE cellulose eluates from rat retina (IC_{50} value 18 ± 6 μM)(Ison, A. J., Johnson, M. S. and Mitchell, R.; unpublished work). Ca^{2+}-independent activity in the fourth fraction, eluting immediately prior to the H7-resistant fraction, was inhibited by H7 with an intermediate IC_{50} value, probably reflecting the presence of small amounts of the H7-insensitive form of PKC in this fraction. All fractions except fraction II contained Ca^{2+}-dependent activity that was sensitive to H7, which is consistent with a previous report that all the cPKCs are sensitive to this inhibitor [38]. As fraction II contained only immunoreactivity for nPKCs but not cPKCs (Figure 3), the absence of Ca^{2+}-dependent activity in this fraction was expected. The Ca^{2+}-dependent activity in fraction V is likely to reflect PKC α, as small amounts of this isoform were detected on immunoblots, although the majority of PKC α was found in fractions III and IV. Of the nPKCs, both δ and ε eluted predominantly in fraction I while θ eluted mainly in fraction III. This elution position for PKC δ is consistent with a report of its co-elution with PKC β [39]. Immunoreactivity for PKC ζ was seen as a doublet of approximately 81 and 88 kDa and was present in all fractions, although only the 88 kDa band was found in fraction V. While the apparent molecular mass of 81 kDa for PKC ζ on SDS-PAGE is consistent with previous reports [40, 41], there is some evidence that the 88 kDa band may be due to cross reaction of this antiserum with another PKC isoform [42]. It is therefore clear that, of PKCs α-θ, only immunoreactivity for PKC α and ζ was present in fraction V, the fraction containing the majority of the H7-resistant activity (Figure 3). The Ca^{2+}-dependent activity detected in this fraction is likely to be due to PKC α but the identity of the kinase responsible for the H7-resistant, Ca^{2+}-independent activity is not known. Since the activity measured in the PKC assay

is evoked by PDBu, it is unlikely that the activity of PKC ζ would be detected because phorbol esters are reported to be inactive on this isoform [9, 10]. Furthermore we have evidence that PKC ζ is detected in our assay as basal (not PDBu-induced) activity and GS peptide, the substrate used in this study, is not a good substrate for this isoform [43]. The possibility remains however that modified states of PKC α-θ could be present, as modification in the region recognised by the antibody may prevent antigen recognition. Furthermore it is possible that PKCs ι (λ) and μ may be present in this fraction although PKC ι (λ) and μ, being aPKCs [7, 8], are unlikely to display activation in the presence of PDBu [4].

In order to detect proteins that may be responsible for the H7-resistant activity, autophosphorylation of HAP fractions was undertaken and the resulting labelled proteins were visualised by autoradiography (Figure 4). In fractions I-III a number of proteins were detected but clear bands were present at molecular masses appropriate for autophosphorylated PKC α and β (95 and 88 kDa respectively). In fraction V from anterior pituitary there was only one clear band, at approximately 140 kDa. This was also present though less prominent in fraction IV but was not seen in the comparable fractions from midbrain. It is therefore possible that this 140 kDa band represents the H7-resistant kinase as its distribution between the pituitary HAP fractions correlates with that of the H7-resistant activity, and this autophosphorylation signal was also detected in the corresponding HAP fractions from lung (unpublished data), another tissue in which a component of the PKC activity is H7-resistant [23]. Immunoblotting of midbrain and pituitary fractions III-IV with a consensus antibody designed to recognise all PKC isoforms showed strong staining of an approximately 130 kDa protein in fractions IV and V from pituitary. Other pituitary fractions and all midbrain fractions failed to show this band. This is consistent with the idea that the 140 kDa phosphorylated protein may represent the H7-resistant PKC itself rather than a coeluting substrate. While this molecular mass is somewhat larger than that of the well characterised PKC isoforms (PKC α-θ) as well as for PKC ι

(λ)[7], reports are now emerging of PKCs that are larger in size. A novel, membrane-bound PKC, PKC μ has been reported in human placenta and several carcinoma cell lines and this isoform is a 117 kDa protein [8]. Furthermore, a 160 kDa form of PKC apparently related to the ζ isoform has been detected in hippocampus by immunoblotting [44]. Some studies have also detected in both hippocampus and platelets, a form of PKC η, designated η', which has a higher molecular mass than the standard PKC η detected in lung and skin (97 kDa rather than 82 kDa)[45, 46]. To verify that this 130 kDa protein may be responsible for the H7-resistant activity, DEAE cellulose eluates of cytosol were fractionated into proteins of molecular mass less than or greater than 100 kDa using centrifugal molecular mass cut-off filters. The PDBu-induced Ca^{2+}-independent PKC activity in the retentate containing proteins with molecular mass greater than 100 kDa was found to contain two components with varying sensitivities to H7. The H7-sensitive activity in this retenate may reflect PKC ε, some of which is likely to be retained by these filters as this isoform has a molecular mass of 94-96 kDa (Figure 3)[47]. The H7-resistant component was inhibited with high potency by the selective PKC inhibitors Ro 31-8220 and GF109203X, providing further evidence that the H7-reistant species is a member of the PKC family with an unusually high molecular mass.

In summary, the H7-resistant kinase previously detected in anterior pituitary tissue can be partially separated from other pituitary PKCs by HAP chromatography. It elutes after the two main peaks of PS-dependent activity, where no activity was detected in other tissues, including midbrain, cerebellum, spleen, mammary gland and COS 7 cells. This elution profile of H7-resistant PKC activity did not correlate with strong immunoreactivity for PKC α-θ though it is conceivable that a modified state of one of these isoforms may not be recognised by the antibodies used. However autophosphorylation revealed a phosphoprotein of approximately 140 kDa which was coincident with and may be responsible for the H7-resistant activity, and a protein of 130 kDa sharing a similar distribution between the fractions was recognised by a PKC consensus antibody. Furthermore ultrafiltration using 100 kDa cut-off filters revealed the H7-

resistant PKC activity to be of a higher molecular mass than other H7-sensitive PKC species (probably greater than 100 kDa). Thus the H7-resistant PKC in anterior pituitary can be distinguished by both size and biochemical properties from the well characterised PKC species and may represent a novel PKC isoform.

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Table 1. Effect of H7 on Ca^{2+}-independent PKC activity in pituitary DEAE-cellulose eluates.

NaCl concentration (mM)	IC_{50} value (μM)
0-50	13 ± 7
50-100	16 ± 3
100-150	77 ± 11
150-200	N.D.

Phosphatidylserine-dependent GS peptide phosphorylation was evoked by 1 μM PDBu at various concentrations of H7 in the absence (<3 nM) of Ca^{2+} and the IC_{50} value determined. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D., not detectable.

Table 2. Effect of H7 on PKC activity in hydroxyapatite fractions from anterior pituitary

HAP fractions	IC_{50} value (μM)	
	Ca^{2+}-independent activity	Ca^{2+}-dependent activity
I	9 ± 2	10 ± 1
II	18 ± 6	N.D.
III	15 ± 8	18 ± 6
IV	45 ± 19	23 ± 5
V	85 ± 10	15 ± 8

Phosphatidylserine-dependent GS peptide phosphorylation was evoked by 1 μM PDBu at various concentrations of H7 in the presence (100 μM free) or absence (<3 nM) of Ca^{2+} and the IC_{50} value determined. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D.,not detectable.

Table 3 Effect of H7 on PKC activity in hydroxyapatite fractions from midbrain

HAP fractions	IC_{50} value (μM)
I	35 ± 5
II	25 ± 7
III	17 ± 4
IV	23 ± 5
V	33 ± 8

Phosphatidylserine-dependent GS peptide phosphorylation was evoked by 1 μM PDBu at various concentrations of H7 in the absence (<3 nM) of Ca^{2+} and the IC_{50} value determined. Values are means ± S.E.M. ($4 \leq n \leq 6$).

Figure 1 Elution profiles of PKC activity from hydroxyapatite column

Tissue extracts were prepared from (a) rat midbrain and (b) rat anterior pituitary and fractionated by HAP chromatography as described in the experimental section. Protein kinase C activity was measured as the amount of phosphorylation of GS peptide (150 μM) phosphorylation in the presence of PS and Ca^{2+} that could be inhibited by PKC a19-31 (pseudosubstrate inhibitor peptide; final concentration 420 μg/ml). Fractions I-V were collected from pituitary fractionation as indicated by the bars and comparable fractions from midbrain were also collected. Typical values for the highest peak of activity were 18 $x10^3$ dpm per assay from midbrain and 12.5 x 10^3 dpm per assay from anterior pituitary (with approximately 2 fold greater specific activity of label). The profiles are representative of those from at least 3 separate experiments in each case.

Figure 2 Inhibition by H7 of PKC activity from anterior pituitary hydroxyapatite fraction V.

Phorbol 12, 13-dibutyrate (1 μM)-induced PKC activity was measured at various concentrations of H7 both in the presence and absence of Ca^{2+} (100 μM and <3 nM free Ca^{2+} respectively). All points are means ± S.E.M. ($4 \leq n \leq 6$). Activity in the presence of Ca^{2+} was stripped of Ca^{2+}-independent activity as described in the Experimental section, to obtain Ca^{2+}-dependent activity. (■) Ca^{2+}-independent, (●) Ca^{2+}-dependent activity

Figure 3 Immunoblots of anterior pituitary hydroxyapatite fractions for PKC isoforms

Anterior pituitary HAP fractions were immunoblotted with antisera specific for PKC α, β_1, δ, ε, θ and ζ as described in the Experimental section. The relative amounts of protein that were loaded onto SDS-PAGE gels for

immunoblotting (the product of sample volume loaded and sample dilution/concentration) were 40:40:1:4:40:40 for anti-PKC α:β1:δ:ε:ζ:θ.

Figure 4 Autophosphorylation autoradiographs of hydroxyapatite fractions III-V from midbrain and anterior pituitary

Autophosphorylation of HAP fractions III-V from midbrain and anterior pituitary was carried out in the presence of PS and Ca^{2+} (5.6 μM), the labelled products being visualised by autoradiography. Phorbol esters were not required to activate PKC under these conditions as there was no detergent present [29]. In pituitary but not midbrain, a strong band was visible at approximately 140 kDa, mainly in fraction V (large arrow). In pituitary fractions III and V and midbrain fraction III, the presumed autophosphorylation of PKC α was visible at about 95 kDa (small arrow).

Figure 5 Immunoblots of hydroxyapatite fractions from anterior pituitary and midbrain with a PKC consensus antibody.

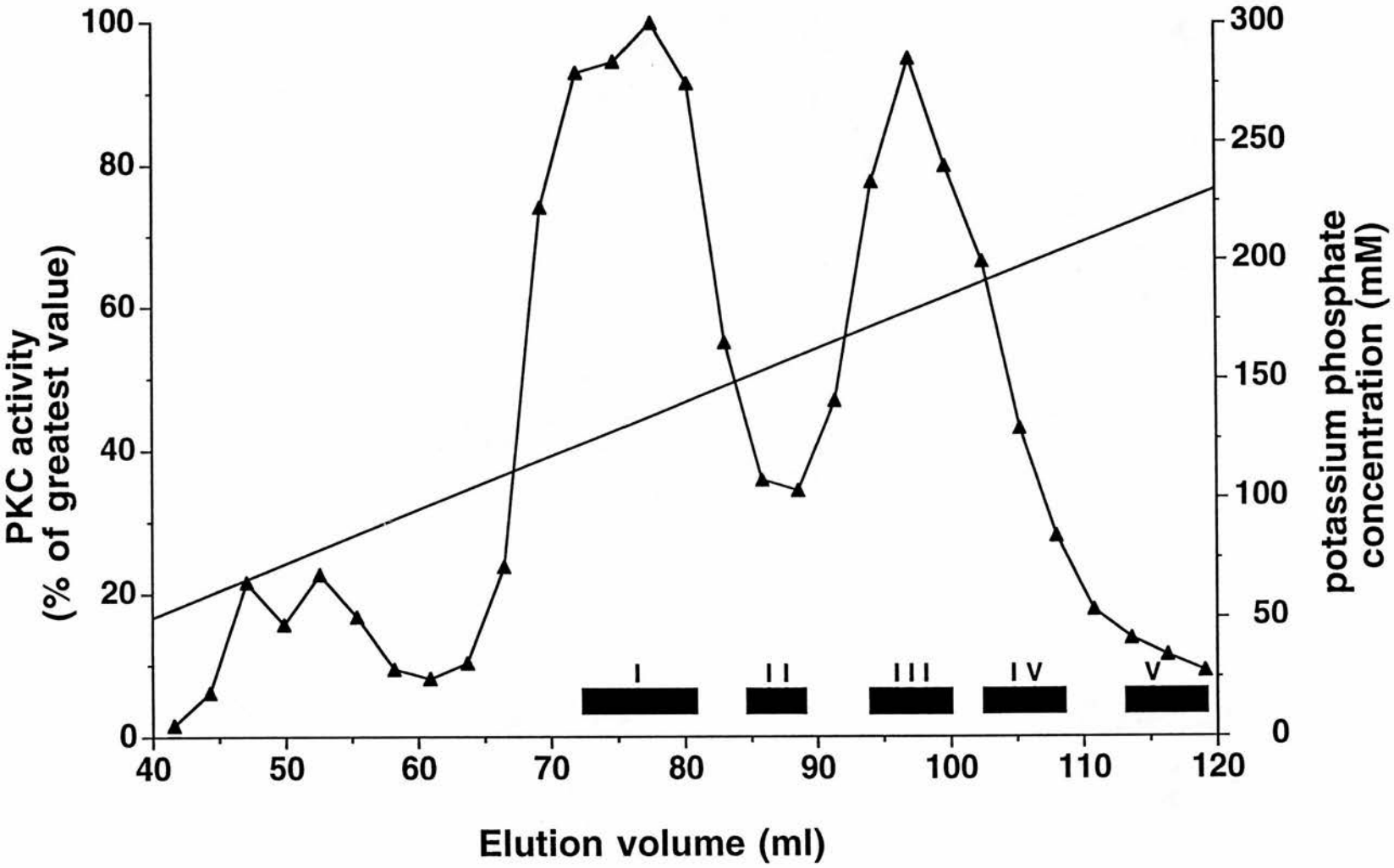
Hydroxyapatite fractions I-V from pituitary and midbrain were immunoblotted with a polyclonal antiserum raised to a PKC consensus sequence in the catalytic domain, PKC α:[Ac 543-550-Cys]. Fractions IV and V from pituitary contained an immunoreactive protein of 130 kDa that was not seen in midbrain or the other pituitary fractions (large arrow). A number of other bands were also apparent in both midbrain and pituitary, including several in the region of 90 kDa (small arrow).

Figure 6 Inhibition by H7 of cytosolic PKC activity separated using 100 kDa molecular mass cut-off filters.

Partially purified cytosolic PKCs from midbrain (a) and anterior pituitary (b) were passed through 100 kDa molecular mass cut off filters and the H7 inhibition of the filtrate, containing proteins less than 100 kDa, and the retentate, containing proteins greater than 100 kDa, were compared. Activity induced by PDBu (1μM) was measured in the absence of Ca^{2+} (<3

nM free Ca^{2+}). All values are means ± S.E.M. ($4 \leq n \leq 6$). Typical values for PDBu-induced activity in the retentate and filtrate from midbrain in absence of inhibitor were 8.3×10^3 and 10×10^3 dpm per assay respectively. Corresponding values from anterior pituitary (with 2 fold greater specific activity of label) were 18×10^3 and 5.7×10^3 dpm per assay respectively.

Figure 1(a)


PKC activity
(% of greatest value)
potassium phosphate
concentration (mM)
Elution volume (ml)
0
20
40
60
80
100
0
50
100
150
200
250
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50
60
70
80
90
100
110
120
I
II
III
IV
V

Figure 1(b)

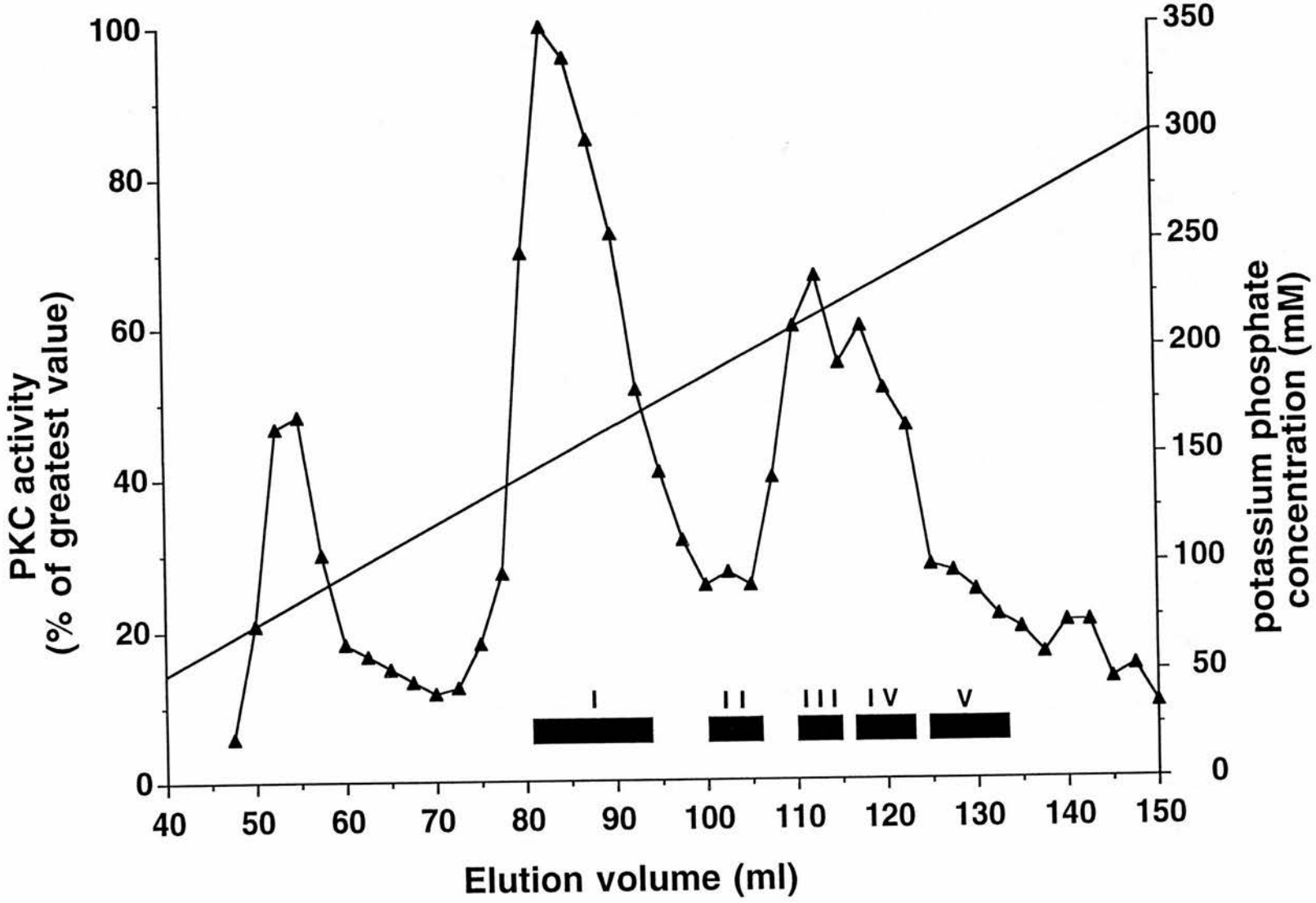

PKC activity
(% of greatest value)
potassium phosphate
concentration (mM)
Elution volume (ml)
100
80
60
40
20
0
350
300
250
200
150
100
50
0
40
50
60
70
80
90
100
110
120
130
140
150
I
II
III
IV
V

Figure 2

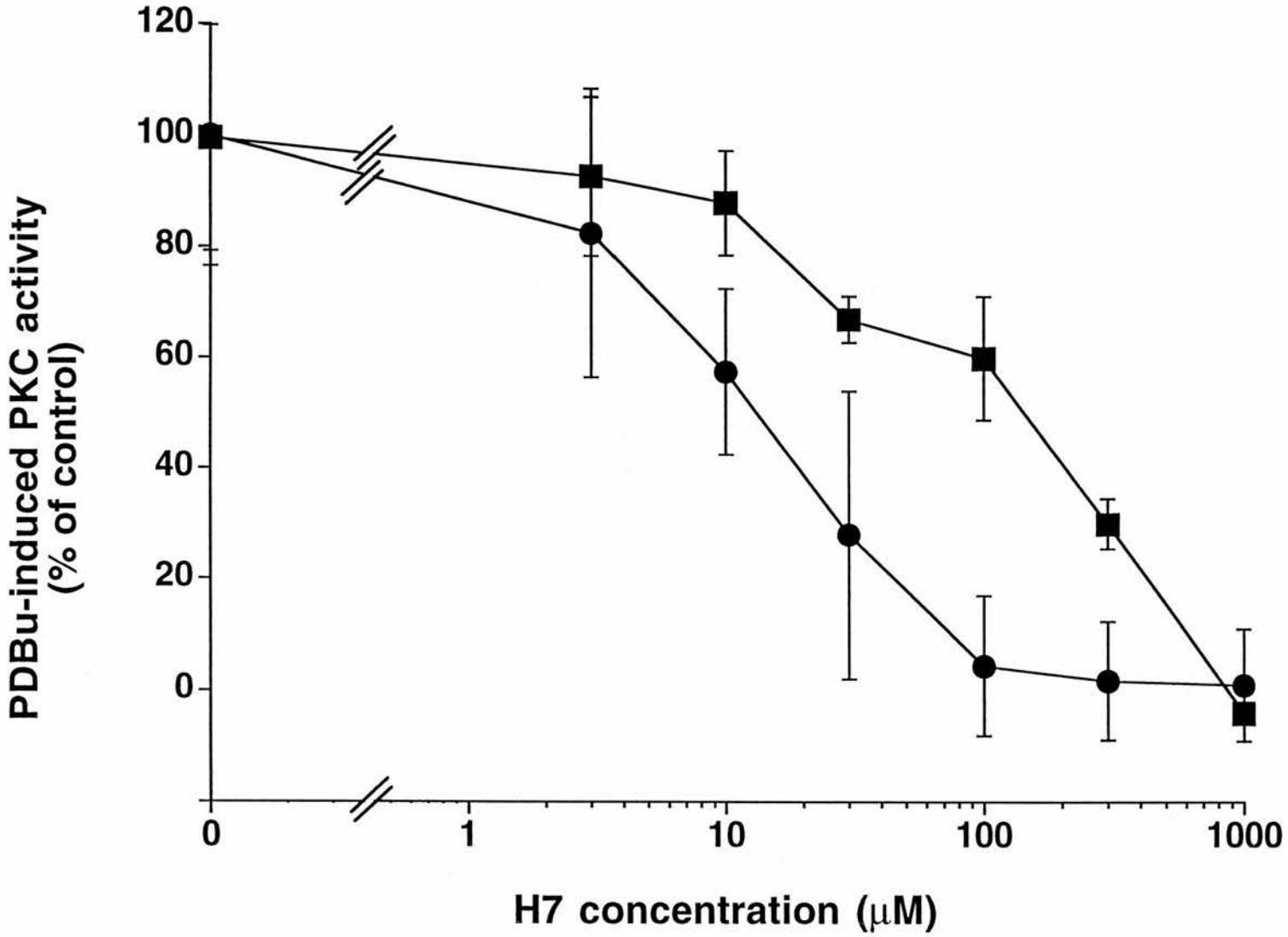

120
100
80
60
40
20
0
PDBu-induced PKC activity
(% of control)
0
1
10
100
1000
H7 concentration (μM)

Figure 6(a)

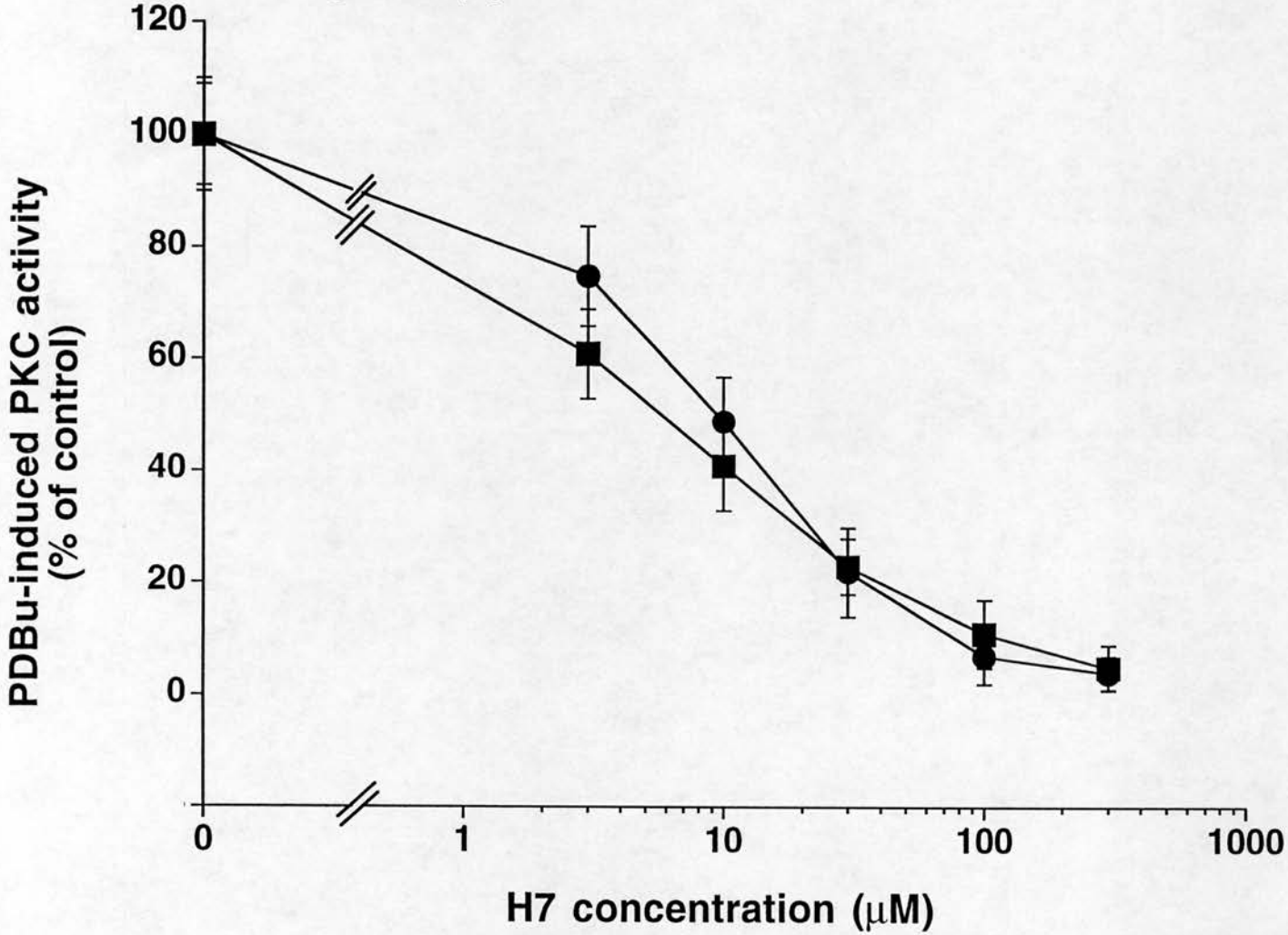
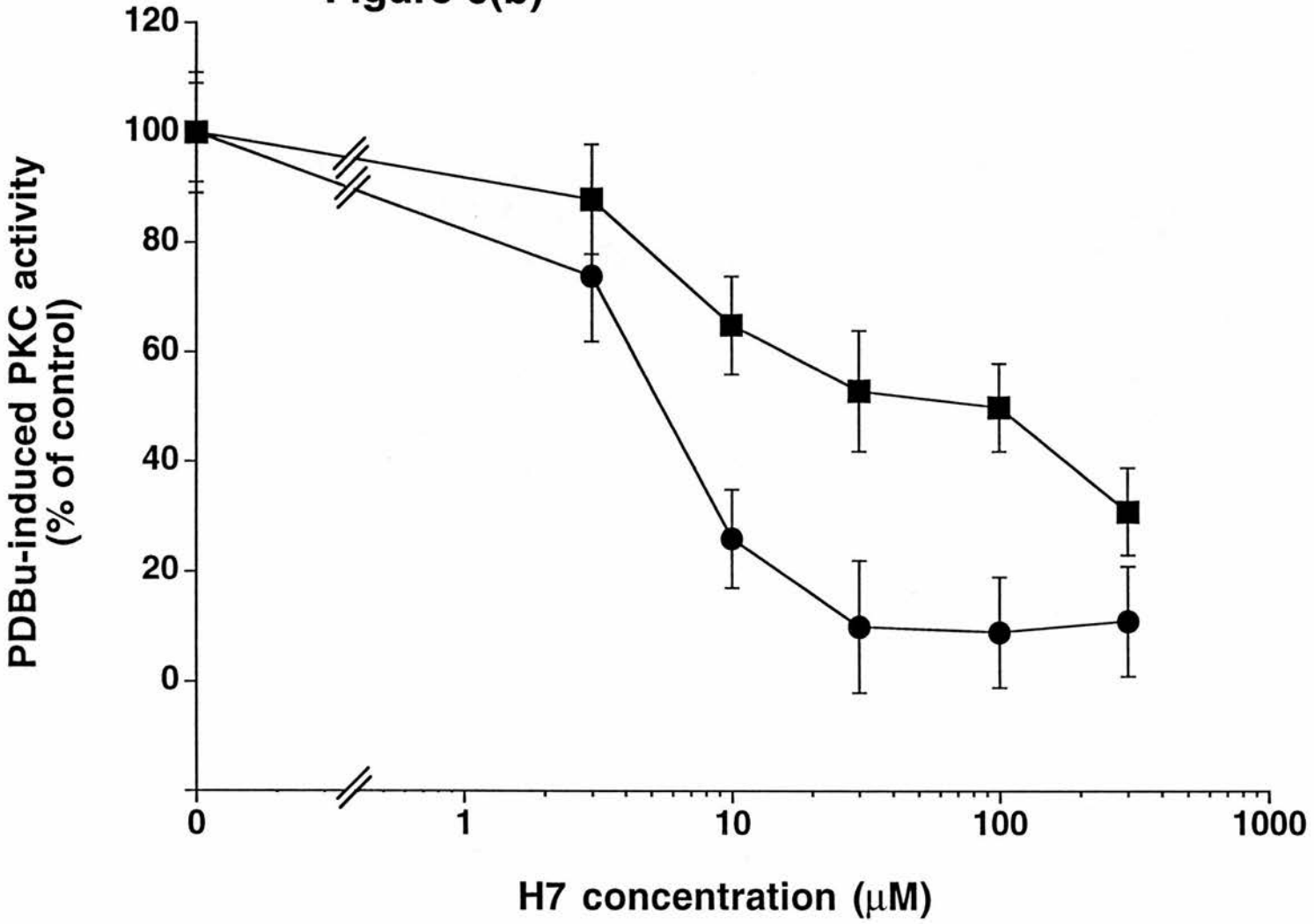

120
100
80
60
40
20
0
PDBu-induced PKC activity
(% of control)
0
1
10
100
1000
H7 concentration (μM)

Figure 6(b)


120
100
80
60
40
20
0
PDBu-induced PKC activity
(% of control)
0
1
10
100
1000
H7 concentration (μM)

Proc. Br. Soc. Cell. Biol. **7**: 172 (1992)

PROPERTIES AND ACTIONS OF A PHORBOL ESTER-STIMULATED BUT H7-RESISTANT HISTONE KINASE IN PITUITARY TISSUE

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We have described a number of cellular responses in anterior pituitary which appear to be mediated by a distinct PKC-like kinase, distinguished by its resistance to isoquinoline but not indolocarbazole or bisindolylmaleimide PKC inhibitors. This kinase appears to be involved in facilitation of 'L'-type Ca^{2+} channel function, secretion of growth hormone, activation of phospholipase A_2 and the self-priming effect of LHRH. Compared to other phorbol-induced responses in similar models, this kinase shows a 6-20 fold reduced potency towards H7 but not staurosporine or Ro31-8220.

A number of putative substrates for this kinase have been identified in [^{32}P] phosphorylation, 2D PAGE experiments, notably 69 kDa, pI 6.1, 36 kDa, pI 6.9 and 16 kDa, pI 6.7.

A mixed micelle kinase assay showed a component of phorbol ester-stimulated, PS-dependent histone III-s kinase activity in pituitary cytosol, which was highly resistant to H7 but not staurosporine. This activity was Ca^{2+}-independent, was eluted from DEAE-cellulose between 0-150 mM NaCl and showed a novel distribution; detected only in anterior pituitary and lung. Northern blots using 3' tailed oligonucleotide probes indicated that mRNA for ε, ζ and perhaps δ PKC are present in pituitary. However, regions rich in δ (thalamus), ξ (PVN), η (skin), α (COS7), β (spleen, cerebral cortex), γ (hippocampus, cerebellum) showed no evidence of H7-resistant activity. It has been reported that α, β, γ and ε are highly H7-sensitive, that γ is absent from anterior pituitary and that of the B series isoforms, ε at least has very low histone kinase activity. This PKC-like kinase may therefore represent a form distinct from the so-far characterised isoforms.

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Evidence for a distinct H7-resistant form of protein kinase C in rat anterior pituitary gland

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Inhibition of phorbol 12,13-dibutyrate-induced protein kinase C (PKC) activity from rat midbrain, anterior pituitary and a number of other tissues, as well as COS 7 cells, was studied in vitro. In anterior pituitary, Ca^{2+}-independent activity was notably resistant to H7 but sensitive to staurosporine and Ro 31-8220. All Ca^{2+}-dependent activity was sensitive to these three inhibitors. Mezerein and 1,2-dioctanoyl-*sn*-glycerol also activated this H7-insensitive PKC from anterior pituitary. The distribution of this activity, prominently expressed in pituitary and perhaps also lung, and its characteristic resistance to H7 but not other inhibitors, does not obviously correlate with that of any of the well-characterised PKCs, and may reflect either a novel or a modified isoform.

Protein kinase C; Pituitary gland; Calcium dependence

1. INTRODUCTION

Protein kinase C (PKC) was originally defined as a phospholipid- and calcium-dependent serine/threonine protein kinase that could be activated by diglycerides and tumour-promoting phorbol esters [1,2]. It is now known that at least 10 structurally different isoforms of PKC exist, which may be subdivided into three groups [3]. The cPKCs (α, βI, βII and γ) all require Ca^{2+} for activation, while the nPKCs (δ, ε, η, θ) and the aPKCs (ζ and λ) all lack the proposed Ca^{2+} binding domain and are active in the absence of Ca^{2+} [3]. Each isoform has been shown to exhibit a unique tissue distribution [4,5]; some (for example δ) being widespread [6], while others, like θ, are highly tissue specific [7], suggesting that each isoform may play a different physiological role. This is supported by studies showing that PKC isoforms vary in their biochemical properties, such as activation by diglycerides and phorbol esters [8], phospholipid dependence [9] and substrate specificity [10]. PKC ζ, for example, is reported not to be activated by phorbol esters and diglycerides [11,12], as this isoform (like λ) lacks one of the cysteine-rich regions that are thought to constitute the high-affinity phorbol binding domain [13]. It is therefore possible that particular PKC isoforms may be activated in response to different cellular stimuli and may phosphorylate distinct target proteins.

In this study, the effect of the inhibitors, staurosporine [14], H7 [15] and Ro31-8220 [16], on cytosolic PKCs partially purified from rat midbrain, anterior pituitary gland and a variety of other tissues, was investigated. The anterior pituitary was assessed because our studies there on the role of PKC in stimulus–secretion coupling revealed participation of a form of PKC that is unusually resistant to H7 but not other PKC inhibitors [17,18]. Midbrain is reported to contain all of the well-characterised PKC isoforms [19], while the other tissues were investigated because of their reported enrichment in particular isoforms. PKC activity evoked by the diterpenes phorbol 12,13-dibutyrate (PDBu) and mezerein, and by the synthetic diglyceride, 1,2-dioctanoyl-*sn*-glycerol (DOG), was studied in cytosol from anterior pituitary and midbrain since certain PKC activators have been shown to differ in their ability to activate individual PKC isoforms in vitro [8] and various PKC-mediated processes in vivo [20–22].

2. MATERIALS AND METHODS

2.1. *Cytosolic PKC activity assay*

Partially purified cytosolic PKC activity was determined as the PDBu-induced histone III-S thiophosphorylation in the presence of phosphatidylserine (PS). All of the activity measured was PS dependent, as it was absent when PS was replaced by phosphatidylcholine which is unable to act as a cofactor in PKC activation [2]. A mixed micelle assay was used in this study to enable the Ca^{2+}-dependent and -independent activity to be analysed separately, as PKC activation is

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obligatorily dependent on phorbol/diglyceride activators under these conditions [23]. The methods used were modified from those in [24,25] and have been briefly described previously [18]. Tissue from male Wistar rats (230–350 g) was homogenised in 2 vols. of 20 mM Tris-HCl (pH 7.5) containing 50 mM 2-mercaptoethanol, 2 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (Sigma), 0.01% (w/v) leupeptin (Sigma) and 20 μM *trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E64) (Sigma). Alternatively, COS 7 cells (cultured at 37°C in DMEM with 10% normal calf serum under 5% CO_2) were washed in Ca^{2+}/Mg^{2+}-free Hank's salt solution and then harvested from tissue culture flasks by scraping into ice-cold homogenization buffer. The suspension was then homogenized using a Ystral polytron homogenizer (Scientific International Industries Ltd., Loughborough, Leics., UK). The homogenate was centrifuged (16.000 × *g*, 20 min, 4°C) and the supernatant was collected and recentrifuged (16.000 × *g*, 5 min, 4°C). The supernatant from the second spin was taken to represent cytosol and was partially purified by loading onto 0.5 ml (cells and pituitaries) or 1.5 ml (tissues) diethylaminoethyl cellulose (DE52: Whatman International Ltd., Maidstone, Kent, UK) in a Bio-Rad Poly-Prep chromatography column (Bio-Rad Laboratories, Richmond, CA, USA) at 4°C. The matrix was then washed with 6 column vols. of homogenization buffer before the partially purified PKC was eluted with 3 column vols. of buffer containing 150 mM NaCl. Cytosolic PKC activity was then measured in an assay mixture (total volume 100 μl) containing, unless otherwise stated, (final concentrations): 10 mM $MgCl_2$, 200 μg/ml PS (sodium salt) (Lipid Products, Nutfield, Surrey, UK), 0.04% Nonidet P-40 (Calbiochem, Novabiochem, Nottingham, UK), 1.25 mg/ml histone III-S (Sigma), 50 μM γ-[^{35}S]ATP (NEN) (0.18 μCi/tube), 1 μM PDBu (LC Services Corp., Scientific Marketing Associates, Barnet, UK) and 25 μl cytosol. PS vesicles were prepared by drying the lipid from chloroform/methanol under a stream of N_2. The subsequent film of PS was scraped into 20 mM Tris-HCl (pH 7.5) with 0.5 mM EGTA, sonicated, then 0.16% Nonidet P-40 was added. The mixture was vortexed before use. Assay tubes also contained either 600 μM $CaCl_2$ (100 μM free Ca^{2+}) or 5 mM EGTA (less than 3 nM free Ca^{2+}) and inhibitors at various concentrations. All assay components and drugs were dissolved in 20 mM Tris-HCl (pH 7.5) with 0.5 mM EGTA except the substrate, histone III-S, which was dissolved in the $MgCl_2$ solution. Reactions were started by the addition of enzyme, incubated at 30°C for 15 min and stopped by quenching with 20 μl 0.1 M ATP in 0.1 M EDTA (pH 7.0). 50 μl of the quenched reaction mixture was spotted onto a 4 cm^2 piece of P-81 cellulose phosphate ion-exchange chromatography paper (Whatman International Ltd.) then washed (3 × 10 ml, 2 min, room temperature) in 75 mM H_3PO_4, dried and counted by liquid scintillation.

2.2. *PKC activators and inhibitors*

PDBu, mezerein (LC Services Corp.), DOG (Sigma), staurosporine (Calbiochem) and Ro31-8220 (Roche Products Ltd. Welwyn Garden City, UK) were made up as 1–10 mM stock solutions in dimethylformamide (DMF). With the exception of DOG (freshly prepared), all of these reagents were used from stocks maintained at −20°C. H7 (LC Services Corp.) was dissolved in distilled water (10 mM) and used from aliquots maintained at −20°C.

2.3. *Purification of PKC α and β*

PKC α and β were more extensively purified from rat brain by hydroxylapatite (Biogel HT, Bio-Rad Laboratories) chromatography following DEAE–cellulose treatment of tissue extracts as described in [26]. Following PAGE the presence of specific immunoreactivity for α and β PKC, respectively, in these preparations was confirmed with specific antipeptide antisera for these isoforms [27]. The specific activities of the preparations used were 17.8 mU/ml and 36.6 mU/ml for PKC α and β, respectively.

2.4. *Data analysis*

Basal activity with PS alone (at appropriate inhibitor concentrations) was subtracted from the inhibitor curves in the absence/presence of Ca^{2+}. A normalised asymmetric sigmoid Hill curve was fitted to the Ca^{2+}-independent evoked activity and subtracted from the evoked activity values obtained in the presence of Ca^{2+}. A normalised curve was then similarly fitted to the resulting values for Ca^{2+}-dependent activity. Curve fitting was carried out using the iterative error-weighted curve fitting program, P.fit (Biosoft, Cambridge, UK).

3. RESULTS

3.1. *Effects of different PKC inhibitors*

Both Ca^{2+}-independent and -dependent PKC activity was elicited in midbrain and anterior pituitary cytosol in a concentration-dependent manner by PDBu (10 nM–3 μM). The response to 1 μM PDBu was almost maximal in each case with typical values for Ca^{2+}-dependent and -independent activity in midbrain of 5.6 ± 0.4 and $12.0 \pm 0.3 \times 10^3$ dpm per mg tissue equivalent. The corresponding values for anterior pituitary were 3.0 ± 1.3 and $3.6 \pm 1.9 \times 10^3$ dpm per mg tissue equivalent. The 1 μM PDBu-induced PKC activity from COS 7 cells was entirely Ca^{2+}-dependent, which is consistent with reports that PKC α is the only phorbol-activated isoform present in these cells [28].

Table I shows IC_{50} values (the concentration required to inhibit 50% of the effect) for inhibition of PKC activity from rat midbrain, anterior pituitary and COS 7 cells by H7, staurosporine and Ro31-8220. In each case, histone III-S thiophosphorylation was stimulated by PDBu (1 μM). In midbrain, staurosporine, Ro-31-8220 and H7 all inhibited both Ca^{2+}-dependent and -independent activity with similar IC_{50} values. The potencies of all three inhibitors on activity from COS 7 cells were similar to those obtained in midbrain. This is in contrast to in anterior pituitary where, although staurosporine

Table I

Effects of H7, staurosporine and Ro31-8220 on PDBu-induced PKC activity from midbrain, anterior pituitary and COS 7 cells

Tissue		IC_{50} (μM)		
		H7	Staurosporine	Ro31-8220
Midbrain	Ca^{2+}-independent	28 ± 5	0.12 ± 0.01	0.18 ± 0.03
	Ca^{2+}-dependent	22 ± 1	0.10 ± 0.04	0.19 ± 0.01
Pituitary	Ca^{2+}-independent	145 ± 38	0.10 ± 0.04	0.34 ± 0.07
	Ca^{2+}-dependent	25 ± 4	0.12 ± 0.05	0.14 ± 0.03
COS 7 cells	Ca^{2+}-dependent	36 ± 11	0.17 ± 0.03	0.26 ± 0.05

PS-dependent histone III-S phosphorylation was evoked by 1 μM PDBu at varying concentrations of inhibitor and the IC_{50} value determined (means ± S.E.M.) ($4 \leq n \leq 6$). Ca^{2+}-independent activity was measured in the presence of EGTA (5 mM) while Ca^{2+}-dependent activity was calculated from the activity in the presence of calcium (100 μM free). There was no detectable Ca^{2+}-independent activity in COS 7 cells.

and Ro31-8220 inhibited Ca^{2+}-dependent and -independent activity with similar potencies. H7 was considerably less potent on Ca^{2+}-independent activity (IC_{50} values of 148 ± 38 and 25 ± 4 μM for Ca^{2+}-independent and -dependent, respectively).

3.2. *Regional differences in the inhibition of PKC*

Table II shows the IC_{50} values for H7 inhibition of PDBu-induced PKC activity from a variety of tissues and cell lines. In the majority of the regions studied, there was no difference in the potency of H7 on the Ca^{2+}-dependent and -independent activity evoked by PDBu, the IC_{50} values varying between 10 and 45 μM. However, in cytosol from anterior pituitary, and perhaps to a lesser extent lung, the Ca^{2+}-independent, but not -dependent, activity was relatively insensitive to H7.

3.3. *The effect of different PKC activators on H7-resistant activity*

The inhibition by H7 of activity evoked by either PDBu (1 μM), mezerein (1 μM) or DOG (1 mM), in midbrain and pituitary preparations is shown in Figs. 1 and 2. At these concentrations both Ca^{2+}-dependent and -independent PKC activity was almost maximal in anterior pituitary (data not shown). In midbrain, H7 was equipotent on Ca^{2+}-dependent and -independent activity evoked by PDBu and DOG (IC_{50} values in the range 22–33 μM) (Fig. 1a and c), although the Ca^{2+}-dependent activity induced by mezerein was somewhat more sensitive to H7 (IC_{50} value 6 ± 1 μM) (Fig. 1b). This is as we have previously reported [29] and is likely to be due to a form of cPKC that is more potently inhibited by H7, retaining rigorous Ca^{2+}-dependence when activated by mezerein. However, in anterior pituitary, PDBu, mezerein and DOG evoked Ca^{2+}-independent activity that was insensitive to H7 (IC_{50} values 145 ± 38, 148 ± 21 and 118 ± 42 μM, respectively) (Fig. 2a). The Ca^{2+}-dependent activity induced by PDBu and DOG in anterior pituitary cytosol was H7 sensitive (IC_{50} values 25 ± 4, 21 ± 10 μM) (Fig. 2b). In anterior pituitary, maximal activity was evoked by mezerein (1 μM) even in the absence of Ca^{2+}, and thus no separate determination of the potency of H7 on Ca^{2+}-dependent mezerein-evoked activity could be made. The maximal amount of PKC activity was similar with both PDBu and mezerein, the only difference being the Ca^{2+}-dependence, which is consistent with previous reports that some activators, like some substrates, do not display rigorous Ca^{2+} dependence [8,9].

Table II

Effect of H7 on cytosolic PKC activity from various sources

Sources	IC_{50} (μM)	
	Ca^{2+}-independent activity	Ca^{2+}-dependent activity
Midbrain	28 ± 5	22 ± 1
Pituitary	148 ± 38	25 ± 4
COS 7 cells	N.D.	36 ± 11
Cerebellum	38 ± 15	34 ± 9
Lung	87 ± 37	39 ± 6
Spleen	19 ± 3	25 ± 3
Liver	15 ± 11	N.D.

PS-dependent histone IIIS phosphorylation was evoked by 1 μM PDBu at various concentrations of H7 in the presence (100 μM free) or absence (< 3 nM) of calcium and the IC_{50} value determined. Values are means ± S.E.M. ($4 \leq n \leq 6$). N.D., not detectable.

3.4. *Inhibition by H7 of α and β isoforms*

Since the cPKC isoforms show some activity in the absence of Ca^{2+} [8], and there is evidence that autophosphorylation of PKC β may diminish its Ca^{2+} dependence [30], it was possible that the H7-resistant Ca^{2+}-independent activity could be due to PKC α or β being activated in the absence of Ca^{2+}. The potency of H7 was therefore determined, in the absence of Ca^{2+}, on PKC α and β, more extensively purified from rat brain by hydroxylapatite (HAP) chromatography. Small quantities of other isoforms may be present within these preparations, but it was evident that both the PKC α and PKC β activities in the absence of Ca^{2+} were sensitive to H7, with IC_{50} values of 35 ± 16 and 40 ± 7 μM, respectively (Fig. 3).

4. DISCUSSION

In this study, the effects of catalytic domain inhibitors on PKC activity partially purified from several tissue and cell line sources have been compared. Staurosporine, H7 and Ro31-8220 are ATP-competitive inhibitors [14–16] but this study has shown differences in their inhibition properties. There is evidence to suggest that, although they all bind close to the ATP site, the precise sites of action appear to be different. H7 has been shown to compete kinetically with ATP [31] but only partially protects the ATP site against denaturation by covalent chemical reagents [32], and this inhibitor displaces Ro31-8220-sensitive the binding of [^{3}H]*N*,*N*-dimethyl staurosporine only at high concentrations [33]. Staurosporine was equipotent on the Ca^{2+}-dependent and -independent activity from midbrain, pituitary and COS 7 cells. This compound is a potent PKC inhibitor but lacks selectivity, also inhibiting other serine/threonine kinases, as well as some tyrosine kinases [34]. The more selective PKC inhibitor, Ro 31-8220 [16], showed a small variation in potency on PKC activity from the sources tested, which is consistent with a recent report [35] that Ro31-8220 shows modest differences in potency between PKC isoforms. The only marked differences in potency, however, occurred with H7; the Ca^{2+}-independent activity from anterior pituitary being particularly insensitive to this inhibitor (Table II). This is consistent with previous reports from this laboratory that a number of pituitary cell functions

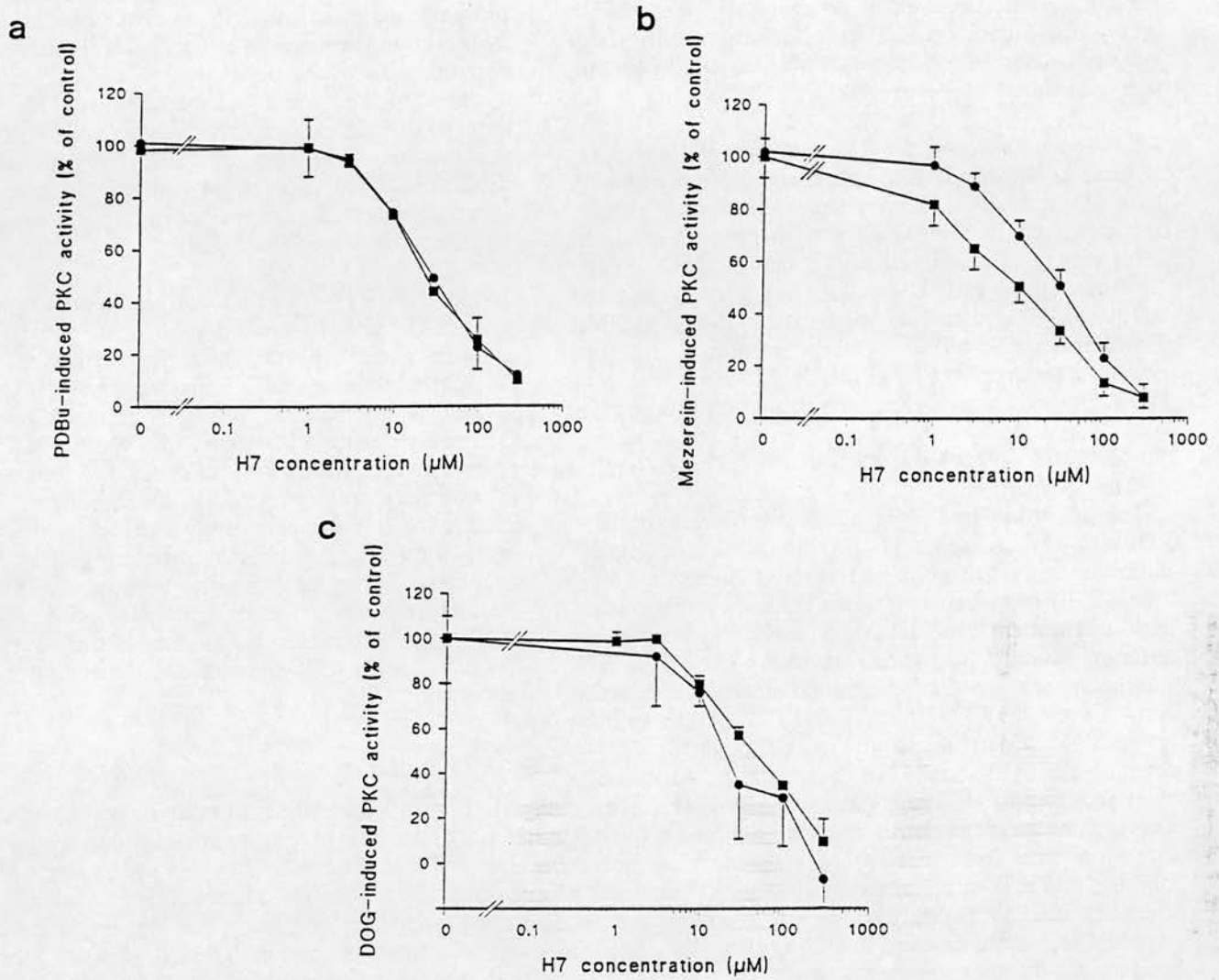

Fig. 1. Inhibition by H7 of (a) PDBu-, (b) mezerein- and (c) DOG- induced PKC activity from midbrain. Histone III-S thiophosphorylation induced by (a) PDBu (1 μM), (b) mezerein (1 μM) or (c) DOG (1 mM) was measured at various concentrations of H7 in the presence of either 100 μM or < 3 nM free Ca^{2+}. All points are means ± S.E.M. ($4 \leq n \leq 6$). IC_{50} values for Ca^{2+}-independent activity were 28 ± 5, 28 ± 4 and 33 ± 13 μM for PDBu, mezerein and DOG, respectively. Activity in the presence of Ca^{2+} was stripped for Ca^{2+}-independent activity as described in section 2, and the IC_{50} values obtained were 22 ± 1, 6 ± 1 and 31 ± 5 for PDBu, mezerein and DOG, respectively. (●) Ca^{2+}-independent activity; (■) Ca^{2+}-dependent activity.

are mediated by an H7-resistant form of PKC [17,18], and from other laboratories describing phorbol ester-induced responses that are inhibited by staurosporine but not H7 [36,37]. When the IC_{50} values for H7 were determined for a number of different tissues, there was little variation in potency on the Ca^{2+}-dependent activity from all the tissues tested, including COS 7 cells where PKC α is the only phorbol--responsive isoform present [28], spleen (containing some α but predominantly β of the cPKCs) [38], and cerebellum (particularly rich in cPKC γ) [38]. This is consistent with previous evidence that the cPKCs do not vary in their response to H7 [39]. The IC_{50} values for Ca^{2+}-independent activity, however, showed wide variations, with pituitary and perhaps lung (but not the other sources tested) containing H7-resistant activity (Table II). Pituitary cells have been shown by immunoblotting to contain PKC α, β, δ, ε and ζ but not the γ or η isoforms [40], while lung has been reported to contain the nPKCs δ, ζ and η but not ε [5,41]. However, Ca^{2+}-independent activity from thalamus and spleen, both containing large amounts of PKC δ [19,42], showed no evidence of H7-resistant activity (Table II), and this isoform, purified from 3Y1 cells, has been reported to be sensitive to H7 [43]. PKC ε, although present in pituitary, is absent from lung [5] and has previously been shown to be H7 sensitive [44]. Furthermore, there is evidence that PKC ε is not able to efficiently phosphorylate histone [44,45]. The other isoform common to both tissues, PKC ζ, is reported not to be activated by phorbol esters [11,12]

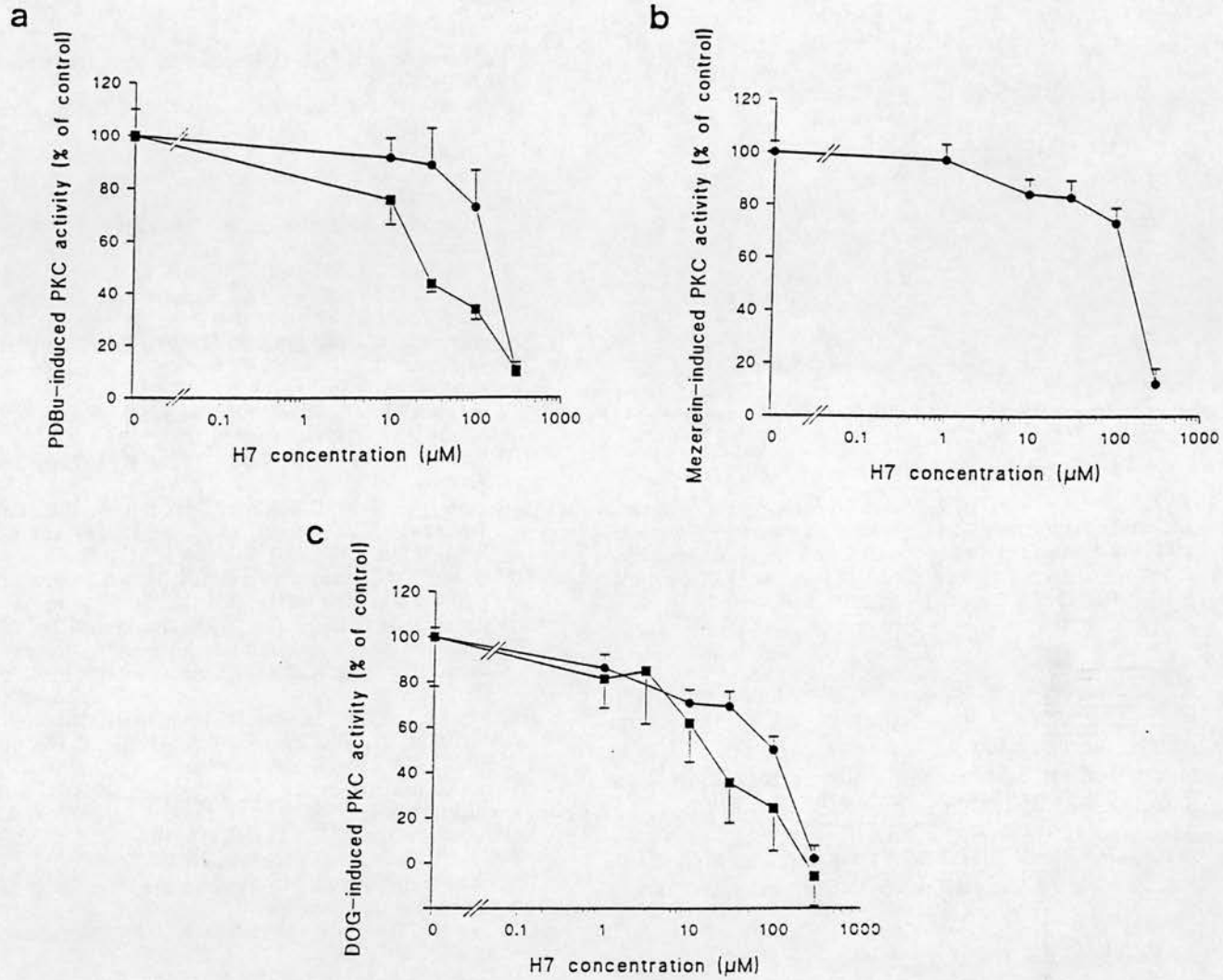

Fig. 2. Inhibition by H7 of (a) PDBu-, (b) mezerein- and (c) DOG- induced PKC activity from anterior pituitary. Histone III-S thiophosphorylation induced by (a) PDBu (1 μM), (b) mezerein (1 μM) or (c) DOG (1 mM) was measured at various concentrations of H7 in both the presence of either 100 μM or < 3 nM free Ca^{2+}. All points are means ± S.E.M. ($4 \leq n \leq 6$). IC_{50} values for Ca^{2+}-independent activity were 145 ± 38, 148 ± 1 and 118 ± 42 μM for PDBu, mezerein and DOG, respectively. Activity in the presence of Ca^{2+} was stripped for Ca^{2+}-independent activity as described in section 2, and the IC_{50} values obtained were 25 ± 4 and 21 ± 10 μM for PDBu and DOG, respectively. Mezerein induced no additional activity in the presence of Ca^{2+}. (●) Ca^{2+}-independent activity; (■) Ca^{2+}-dependent activity.

and is also present in COS 7 cells [46] which showed no PDBu- induced Ca^{2+}-independent activity. Thus the tissue distribution of this H7-resistant PDBu-induced activity clearly does not correspond to any of these nPKC or aPKC isoforms.

PKC activators other than PDBu have been shown to elicit quite different effects on $^{45}Ca^{2+}$ influx through L-type Ca^{2+} channels in the anterior pituitary compared to the GH_3 cell line [20,21], and to differ in their ability to activate individual PKC isoforms in vitro [8]. This study has shown that both mezerein and DOG were also able to evoke H7-insensitive Ca^{2+}-independent PKC activity in pituitary cytosol (Fig. 2), consistent with the ability of these compounds to selectively activate an H7-resistant form of PKC which facilitates Ca^{2+} entry through L channels [23,24]. It has been reported that certain diterpenes are capable of activating cPKCs in the absence of Ca^{2+} [8], but there is evidence that DOG is only able to activate PKC α and possibly β in the presence of Ca^{2+} ([47] and Johnson et al., unpublished observations). Thus the ability of DOG to elicit the H7-resistant kinase activity suggests that this is not due to Ca^{2+}-independent cPKC activity. This was further substantiated when the inhibition by H7 of PKC α and β purified from rat brain by HAP chromatography were studied in the absence of Ca^{2+} and shown to be of normally high potency.

Thus it appears that the H7-insensitive kinase, found in pituitary and perhaps lung does not correspond to any of the well-characterised Ca^{2+}-independent iso-

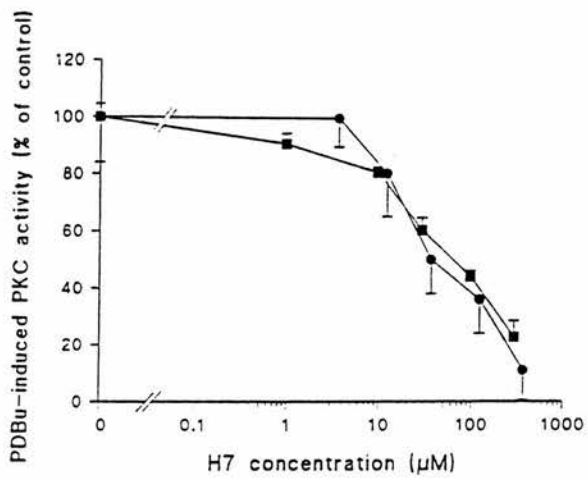

Fig. 3. Inhibition by H7 of PKC α and β purified from rat brain by HAP chromatography. PDBu (1 μM)-evoked PKC activity was measured in the presence of < 3 nM free Ca^{2+} with varying concentrations of H7. All points are means ± S.E.M. ($n = 4$). (●) PKC α; (■) PKC β. IC_{50} values for PKC α and β were 35 ± 16 and 40 ± 7 μM, respectively.

forms and is not a cPKC activated in the absence of Ca^{2+}. This activity may represent one of the incompletely characterised isoforms, such as PKC θ or λ; alternatively it may be a modified form of one of the known PKCs or perhaps a novel PKC isoform.

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CHARACTERIZATION OF AN H7-RESISTANT PROTEIN KINASE C FROM ANTERIOR PITUITARY CELLS. A.J. Ison, E. Lutz, M.S. Johnson, D.J. MacEwan and R. Mitchell, MRC Brain Metabolism Unit, 1 George Square, Edinburgh, EH8 9JZ, UK.

It has previously been shown that a variety of anterior pituitary cell functions, including LHRH priming, are mediated by a calcium-independent form of protein kinase C (PKC) that is unusually resistant to the PKC inhibitor, H7. To further characterize this kinase, a mixed-micelle assay was used to measure the phosphatidyl serine-dependent kinase activity of partially-purified cytosol in the absence of calcium. In anterior pituitary, phorbol 12,13-dibutyrate (PDBu)-induced phosphorylation of histone IIIS was relatively insensitive to H7 (IC_{50} 154 ± 42 μM) but sensitive to staurosporine and Ro 31-8220 (IC_{50}s 117 ± 46 and 295 ± 84 nM, respectively). PDBu-induced activity in many other tissues was sensitive to H7 (eg midbrain, IC_{50} 27± 9 μM). Mezerein, but not 1,2 dioctanoyl-*sn*-glycerol, also induced H7-resistant activity in pituitary (IC_{50}s 177 ± 27 and 23 ± 9 μM, respectively). We investigated the possible involvement of B-series PKC isoforms. Northern blots showed that anterior pituitary contains ε and ζ but provided no evidence for δ. Kinase activity in thalamus and intestine (δ-rich) and PVN of the hypothalamus (ζ-rich) was sensitive to H7. PKCε is reported to be H7-sensitive and to efficiently phosphorylate myelin basic protein (MBP) but not histone IIIS. PDBu-induced MBP phosphorylation in anterior pituitary cells showed no H7 resistance (IC_{50} 46 ± 12 μM). These results suggest that this H7-insensitive kinase is unlikely to be PKC δ, ε or ζ.

Biochemical characterisation of an apparently novel isoform of protein kinase C in pituitary

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The protein kinase C (PKC) family of phospholipid-dependent serine/threonine protein kinases is now known to contain at least ten structurally different members [1]. These isoforms may be subdivided into two groups, the cPKCs (α, βI, βII and γ) which are dependent on calcium for activation and the nPKCs (δ, ε, ζ, η, θ, and λ) which are active in the absence of calcium. Each isoform has been shown to have a unique tissue distribution [2, 3] and to vary in their biochemical properties such as substrate specificity [4], phospholipid dependence [5] and activation by different tumour promoters [6]. It is therefore likely that they may play different roles in signal transduction.

We have previously shown that a variety of anterior pituitary cell functions are mediated by a form of PKC that is unusually resistant to the PKC inhibitor H7 but sensitive to other inhibitors such as staurosporine and Ro31-8220 [7, 8, 9, 10]. To further characterise this form of PKC, we used a mixed micelle assay to measure PKC activity in cytosol from various tissues, partially-purified on DEAE cellulose columns as described in [8]. Phorbol 12, 13-dibutyrate (PDBu)-induced phosphatidylserine (PS)-dependent histone IIIS thiophosphorylation was determined in both the presence and absence of Ca^{2+}. In cytosol from the anterior pituitary, the Ca^{2+}-independent but not Ca^{2+}-dependent activity was relatively insensitive to H7 (IC_{50} values 148 ± 40 and 25 ± 4 μM respectively). The other tissues investigated, including midbrain, cerebellum, spleen and liver showed no evidence of H7-resistant PS-dependent activity (IC_{50} values ranging from 15-43 μM). When [$Ala^{9,10}$, $Lys^{11,12}$] glycogen $synthase_{1-12}$ (GS peptide) but not $[Ser]^{25}$ PKC α_{19-31} was the phosphate acceptor, a clear component of the activity, which was Ca^{2+}-independent, was resistant to H7 (IC_{50} value 71 ± 11 μM).

To further purify this species of PKC, DEAE-cellulose chromotography of pituitary cytosol was followed by hydroxylapatite (HAP) chromatography as described in [11]. This method has previously been shown to resolve PKC from brain into 3 separate peaks of activity containing either PKC α, β or γ, although these will also contain the nPKCs. The PS-dependent PKC activity in pituitary cytosol was resolved into two main peaks eluting at 95-135 mM and 165-190 mM potassium phosphate but there was also PKC activity eluting at a higher phosphate concentration (200-220 mM) that was not present in cytosol from other sources including brain, spleen and COS 7 cells. Fractions were collected as indicated and immunoblotted for the α, β, δ, ε and ζ isoforms of PKC. The numbers in parentheses represent the apparent molecular weight (kDa) of the predominant component of the doublet detected specifically by the ζ antibody.

fraction	potassium phosphate concentration (mM)	isoforms present
I	95-135	β, δ, ε, ζ (80)
II	140-155	δ, ε, ζ (80)
III	165-175	α, ζ (85)
IV	180-190	α, ζ (85)
V	200-220	some ζ (85)

Inhibition of the PDBu-induced PS-dependent GS peptide phosphorylation activity of these fractions showed H7 resistant, Ca^{2+}-independent activity in fraction V (IC_{50} value 83 ± 17 μM) but not in fractions I-III (IC_{50} values 9-18 μM). The IC_{50} value of the Ca^{2+}-independent activity in peak IV was intermediate between these values (45 ± 19 μM), probably reflecting a combination of H7-sensitive and resistant PKCs. All Ca^{2+}-dependent activity was H7-sensitive (IC_{50} values 10-23 μM) and all activity in fractions I-V was inhibited by Ro 31-8220 (IC_{50} values 103-169 nM).

Thus these studies have shown that it is possible to partially purify the H7-resistant PKC from other isoforms present in pituitary. The anterior pituitary has been shown by immunoblotting to contain the α, β, δ, ε and ζ isoforms of PKC but not PKC γ [12, 13]. The H7-insensitive kinase is unlikely to represent PKCδ-ζ as it elutes from HAP columns at a position not coincident with strong anti δ-ζ immunoreactivity. Furthermore, PKC δ and ε have previously been reported to be H7-sensitive [14, 15] and PKC ζ is not thought to be activated by phorbol esters [16, 17]. The H7-resistant PKC may represent one of the less-characterised isoforms such as η, θ or λ, a modified form of a PKC or possibly a novel isoform.

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BIOCHEMICAL AND PHARMACOLOGICAL EVIDENCE FOR A DISTINCT FORM OF PROTEIN KINASE C SELECTIVELY EXPRESSED IN PITUITARY.

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We have previously shown in a variety of physiological models of anterior pituitary cell function, including LHRH self priming and regulation of L-type Ca^{2+} channels, the involvement of a form of protein kinase C (PKC) that is unusually resistant to the kinase inhibitor, H7. To further investigate this *in vitro*, a mixed micelle assay was used to measure phorbol 12, 13-dibutyrate (PDBu)-induced, phosphatidyl serine-dependent kinase activity . In partially-purified cytosol from anterior pituitary but not midbrain, Ca^{2+}-independent activity was relatively insensitive to H7 (IC_{50} values 148 ± 40 and 28 ± 5 μM respectively), though in both cases Ca^{2+}-dependent activity was H7 sensitive (IC_{50} values 25 ± 4, 22 ± 1 μM respectively). Fractionation by hydroxylapatite chromatography revealed that cytosol from pituitary but not midbrain contained PDBu-induced kinase activity eluting after the positions of PKC α-ζ, beyond the main peak of PKC α activity. H7 inhibition profiles showed that the Ca^{2+}-independent activity in this fraction but not in fractions containing the majority of PKC α activity or in those eluting prior to this, was H7-resistant (IC_{50} value 83 ± 17 μM compared to a range of 9-20 μM in the other fractions). All Ca^{2+}-dependent activity was H7 sensitive (IC_{50} values 10-23 μM). PDBu-induced activity in all fractions was sensitive to the PKC inhibitor Ro31-8220 (IC_{50} values < 200 nM). This H7-insensitive kinase found in the anterior pituitary may represent a novel isoform of PKC or another PKC-like kinase.

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UNCONVENTIONAL SIGNALLING BY THE LHRH RECEPTOR

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The LHRH receptor is a member of the family of G protein-linked receptors coupled to hydrolysis of phosphoinositides [1]. The dependence of LHRH-induced gonadotrophin secretion upon phospholipase C activity has however been questioned [2]. In view of the unique ability of the LHRH receptor to elicit the phenomenon of self-priming [3], we have sought to define unconventional signals emanating from this receptor that may not be generated by other members of the receptor family.

The phenomenon of LHRH self-priming is dependent upon an apparently novel species of PKC with a pituitary-selective distribution [4] that we have partially purified and characterised [5]. Amongst the cellular targets of a PKC with these properties are PLA_2 and PLD.

Signalling by means of tyrosine kinase and MAP kinase cascades is classically associated with growth factor receptors. Nevertheless we have shown that each of these is strongly activated by LHRH both in normal pituitary tissue and in the αT3-1 gonadotroph cell line. LHRH-induced production of phosphotyrosine-immunoreactive proteins is mimicked in part by phorbol esters but not by ionomycin and is essential for LHRH-induced PLD activation and LH secretion [6]. LHRH-induced MAP kinase activation correlates strongly with the ability of gonadotrophs to demonstrate self-priming and occurs through a PKC-dependent rather than Ca^{2+}-dependent or tyrosine kinase-dependent mechanism [7]. Since MAP kinase is involved in the regulation of transcriptional, translational and cytoskeletal organisation, it is a strong candidate for a central role in the self-priming phenomenon which is reliant on the rapid induction of protein synthesis and cytoskeletal changes.

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A novel high molecular weight form of protein kinase C in anterior pituitary

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The protein kinase C (PKC) family of phospholipid-dependent serine/threonine kinases has been implicated in a wide variety of cellular processes, including growth, differentiation, control of gene expression and secretion of hormones [1]. There are now known to be at least eleven structurally different isoforms, which may be divided into subgroups on the basis of their activation characteristics; the Ca^{2+}-dependent cPKCs (α, β_1, β_2 and γ), the Ca^{2+}-independent nPKCs (δ, ε, η and θ) and atypical aPKCs (ζ, ι and μ), the activity of which, unlike the other two groups is reported to be unaffected by phorbol esters. The eleven isoforms have been shown to differ widely in their substrate specificity and phospholipid dependence [2] as well as in their tissue and cellular localisation [3, 4].

We have previously shown in the anterior pituitary that a number of physiological processes are mediated by a form of PKC which, although sensitive to the PKC inhibitors staurosporine and Ro 31-8220, is unusually resistant to another PKC inhibitor H7 [5, 6, 7]. This kinase, detectable in anterior pituitary cytosol using a mixed-micelle PKC activity assay, is activated by phorbol esters in the absence of Ca^{2+} but does not appear to correspond to any of the well-characterised PKC isoforms [8] and can be biochemically separated from some of the other pituitary PKCs by hydroxyapatite (HAP) chromatography [9]. On HAP fractionation of pituitary tissue extracts, the H7-resistant kinase activity elutes after the main peak of PKC α in a region where no activity is seen in other tissues. To detect proteins that may be responsible for this H7-resistant kinase activity, five HAP fractions from anterior pituitary were collected and allowed to autophosphorylate in the presence of phosphatidylserine and Ca^{2+} with ATP-γ[^{32}P]. The resulting phosphorylated proteins were visualised by autoradiography. The fractions containing H7-resistant activity (fractions IV and V, both eluting after the main peak of PKC α) contained one predominant phosphoprotein with a molecular mass of approximately 140 kDa. This protein was not detected in fractions I-III (corresponding to the main peaks of PKC α and β, and the region between these two peaks). Immunoblotting with an antiserum raised to a sequence conserved between all the PKC isoforms (PKC α: [Ac 543-550-Cys]) showed corresponding immunoreactivity of a 130 kDa protein, which was strongly detected in fractions IV and V. Small amounts of immunoreactivity was also visible in fraction III but not in any of the other fractions.

To assess whether the H7-resistant kinase may be a modified version of one of the well-characterised PKC isoforms, fractions were immunoblotted with antisera specific for PKC α-θ. This showed that only PKC α and ζ were present in fractions IV and V, and no specific immunoreactivity >100 kDa was detected. Protein kinase C α is unlikely to be responsible for the H7-resistant activity detected in these fractions as this isoform is dependent on Ca^{2+} for activation while the H7-resistant activity found in anterior pituitary is Ca^{2+}-independent. Furthermore PKC α purified from brain was sensitive to H7 in both the presence or absence of Ca^{2+} (IC_{50} values 13 ± 6 and 18 ± 8 μM respectively). The other isoform detected in fractions IV and V, PKC ζ, is reported to be active in the absence of phorbol esters [10] and we have evidence that this isoform is detected in our assay as basal activity [11], unlike the H7-resistant activity which is PDBu-evoked. Thus the 130 kDa protein may well represent the H7-resistant kinase. To investigate the possibility of an extended PKC ζ-like species which may be able to bind phorbol esters, a PCR approach was employed. PKC ζ and ι (λ), unlike the phorbol-activated PKC isoforms, contain only one cysteine rich region in the C1 domain, thought to contain the phorbol ester binding site, so a modified form containing an extra sequence in this domain might be phorbol-responsive. Therefore oligonucleotide primers were designed from the rat PKC ζ sequence corresponding to the regions flanking C1 domain. However PCR amplification from a rat pituitary cDNA library resulted in only one product of 577 base pairs, the size expected for PKC ζ, and when the 5 primer was paired with a primer corresponding to a conserved region in the protein kinase domain of PKC, only the predicted 933 base pair fragment was amplified.

Thus these studies have shown that the H7-resistant PKC activity found in anterior pituitary coelutes from HAP with a 140 kDa phosphoprotein (as detected by autophosphorylation) and which also appears to be recognised by a PKC consensus antiserum. While this size is larger than that of the well-characterised PKC isoforms (PKC α-θ), there are recent reports of larger forms of PKC. A novel membrane-bound PKC isoform, PKC μ has a molecular mass of 117 kDa [12] and there are also reports of high molecular mass forms of PKC η and ζ in platelets and hippocampus respectively [13, 14]. Since we were unable to detect an extended form of PKC ζ by PCR, or any high molecular mass immunoreactivity for PKC ζ and η, and PKC μ is reported to be unreponsive to phorbol esters, it would appear that this H7-insensitive kinase may represent a novel high molecular mass form of PKC.

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